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

II. COMPLETE POLYPEPTIDE CHAIN COMPOSITION INVESTIGATED BY MAXIMUM ENTROPY ANALYSIS OF MASS SPECTRA*

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The deep-sea tube worm Riftia pachyptila J ones possesses a complex of three extracellular Hbs: two in the vascular compartment, V1 (~3500 kDa) and V2 (~400 kDa), and one in the coelomic cavity, C1 (~400 kDa). These native Hbs, their dissociation products and derivatives were subjected to electrospray ionization mass spectrometry (ESI-MS). The data were analyzed by the maximum entropy deconvolution system. We identified three groups of peaks for V1 Hb, at ~16, 25-27, and 30 kDa, corresponding to (i) two monomeric globin chains, b (M, 16,133.5) and c (M, 16,805.9); (ii) four linker subunits, L1-L4 (M, 23,505.2, 23,851.4, 26,342.4, and 27,425.8, respectively); and (iii) one disulfide-bonded dimer D1 (M, 31,720.7) composed of globin chains d (M, 15,578.5) and e (M, 16,148.3). V2 and C1 Hbs had no linkers and contained a glycosylated monomeric globin chain, a (M, 15,933.4) and a second dimer D2 (M, 32,511.7) composed of chains e and f (M, 16,368.1). The dimer D1 was absent from C1 Hb, clearly differentiating V2 and C1 Hbs. These Hbs were also subjected to SDS-PAGE analysis for comparative purposes. The following models are proposed for the quaternary structure of the Hbs: (a) (cD)(bD)6(aD) (D corresponding to either D1 or D2) for V1 and C1 Hbs. HBL V1 Hb would be composed of 180 polypeptide chains with 144 globin chains and 36 linker chains, providing a total molecular mass ~3285 kDa. V2 and C1 would be composed of 24 globin chains providing a total molecular mass ~403 kDa and 406 kDa, respectively. These results are in excellent agreement with experimental M, determined by STEM mass mapping and MALLS (8).

Several models have been proposed for the quaternary structure of annelid hexagonal bilayer hemoglobins (Hbs)1-5 and vestimentiferan Hbs6 but no definitive agreement exists. However, it is well established that HBL Hbs have a hexagonal symmetry and consist of two types of chains, globin-chains (~17,000 Da) accounting for approximately 70% of total mass and heme-deficient linker chains (~24,000–32,000 Da) necessary for assembly into the HBL structure. A detailed understanding of this molecular structure requires the knowledge of: (i) the molecular mass of the native molecule; (ii) the number and proportions of all constitutive polypeptide chains; (iii) the number and relation between subunits; and (iv) the determination of linker proportions.

In a companion study (8) we have confirmed that Riftia pachyptila (J ones), a vestimentiferan living around deep-sea hydrothermal vents (9-11), possesses three hemoglobins, two of them dissolved in the vascular blood (V1 and V2), and one in the coelomic fluid (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 mass significantly higher than the literature data for V1. V1, V2, and C1 had Mr of 3396 ± 540 × 10^3, 393 ± 71 × 10^3, and 410 ± 51 × 10^3 by STEM, and 3503 ± 13 × 10^3, 433 ± 8 × 10^3, and 380 ± 4 × 10^3 by MALLS, respectively. However, STEM mass mapping measurements for V1 Hb were actually made on one-twelfth molecules (8) and the M, noted above is therefore an approximation, probably less accurate than MALLS result. Transmission electron micrographs of V1 are typical of a hexagonal bilayer hemoglobin (HBL Hb).

Reduced annelid Hbs exhibit a variety of SDS-PAGE profiles, ranging from one subunit of Mr 13,000–15,000 to seven subunits in the range of Mr 13,000 to 40,000 (12). More recently, a more accurate technique, ESI-MS, has been successfully applied to some HBL Hbs allowing the determination of the complete polypeptide chain compositions of these Hbs (13-15). They consist of 4 to 6 globin chains in the range 16,000–18,000 Da and 3-4 linker chains in the range 24,000–27,000 Da. Previous studies by SDS-PAGE on Riftia vascular Hbs have shown that they consist of subunits of Mr ~15,000, 30,000, and 45,000 (16-18). The amino acid compositions of these Hbs are very similar to each other and they contain 1 heme per 23,000 g of protein as in annelid Hbs (17).

The aim of the present work was to conceive realistic models of the quaternary structure of Riftia heteromultimeric Hbs. In a companion study (8) we have determined the precise masses of three Riftia Hbs. In this report we have determined the stoichiometry of the polypeptide chains and subunits constituting these Hbs by ESI-MS coupled with maximum entropy (19).
In addition, these results are compared with those obtained by SDS-PAGE.

**EXPERIMENTAL PROCEDURES**

Animal Collection—Live R. pachyptila were collected at about 2600 m depth during four oceanographic cruises (October 1991, April 1992, November 1994, and April 1995) with the "Nautilus" and "Alvin" submersibles on the East Pacific Rise at 13°N (12°46'–12°50'N and 103°57'–103°56'W) and 9°N (9°48'–9°50'N and 104°17'W). Specimens which had not been damaged during collection were dissected 2 to 8 h after capture and the fluids contained in their circulatory and coelomic compartments were separately and carefully collected and stored as described previously (20).

**Purification Techniques—**Hemoglobins were separated by gel filtration on a 1 × 30 cm Superose 6-C column (Pharmacia Biotech Inc.) using a low pressure fast protein liquid chromatography system (Pharmacia). The column was equilibrated with a Rittia saline buffer as described in Zal et al. (8) and concentrated with microconcentrator Centricon-10 (Amicon). One or two further purifications using the same protocol were performed to obtain clear fractions when necessary.

SDS-PAGE—SDS-PAGE was carried out on the different Hbs using the discontinuous buffer system of Laemmli on slab gels (21). The gel dimensions were 1.5 mm × 16 cm × 18 cm (LK8 instrument). The stacking gel was constituted of 4.2% of acrylamide and bis-acrylamide, pH 6.8, and the resolving gel by 10 and 2.7%, pH 8.8.

Purified Hb samples were placed on top of a new gel and electrophoresed as described above. The gels were stained overnight on agitator in 0.125% Coomassie Brilliant Blue R-250 (Bio-Rad) in 50% methanol, 10% acetic acid, water. The molecular mass of each constituent polypeptide chain was determined from a plot of the log molecular mass versus the mobility of low molecular weight protein standards of Pharmacia (22). The gels were scanned at 550 nm on a Vernier densitometer and the peaks area were determined by a Coradi planimeter.

**Electrophoresis with SDS—**PAGE—The unreduced Hb V1 dissociated in SDS into six subunits (lane 1): subunits A1 and A2 with apparent molecular weights of 16,000 and 17,000, subunit A3 with M, 29,000, subunits A4 and A5 both with M, close to 32,000 and subunit A6 with M, 36,000. After reduction (lane 2), Hb V1 dissociated into eight polypeptide chains consisting of two groups: one in the range M, 16,000–19,000 (AI–AIV) and the other in the range M, 29,000–40,000 (AV–AVIII). Subunits A1 and A2 correspond to the monomeric globin chains AI1 and AIV, subunit A3 would be a disulfide-bonded dimer of globin chains A1 and A11, and subunits A4 to A6 correspond to four non-heme-containing chains AV–AVIII.

V2 and C1 have similar dissociation patterns with three subunits of M, 16,000, 19,000 and 29,000: B1, B2, and B3 (lane 3), and C1, C2, and C3 (lane 5), respectively. Upon reduction V2 and C1 dissociate into four polypeptide chains with M, ranging from 16,000 to 19,000: BI to BIV (lane 4) and C1 to ClV (lane 6), respectively. Subunits B3 and C3, like subunit A3 in V1 Hb, correspond to disulfide-bonded dimers of globin chains B1 and B11, and C1 and C11, respectively.

**Polypeptide Chains Composition Determined by ESI-MS—**

The nomenclature used to name the different subunits and polypeptide chains was that previously used for Lamellibrachia sp. (6). Fig. 1 presents the SDS-PAGE profiles of R. pachyptila Hbs V1 (lanes 1 and 2), V2 (lanes 3 and 4) and C1 (lanes 5 and 6). Lanes 1, 3, and 5, unreduced Hb; lanes 2, 4, and 6, reduced Hb. Relative molecular weights (M) were estimated using low molecular weight protein calibrants (Pharmacia).

**RESULTS**

**Polypeptide Chains Composition Determined by SDS–PAGE—**

The nomenclature used to name the different subunits and polypeptide chains follows the one used for Lumbricus terrestres HBL Hb (28). Fig. 2A shows the raw, multicharged ESI mass spectrum of HBL Hb V1. Fig. 2B–D, shows deconvoluted spectra obtained by MaxEnt analysis for Hbs V1, V2, and C1. The deconvoluted mass spectrum of V1 Hb (Fig. 2B) revealed three groups of components. The first one around M, 16,000–17,000, comprised two polypeptide chains with M, 16,133.5 ± 0.7, and c, M, 16,805.9 ± 0.8. The middle group, around M, 23,000–27,000, comprised two major components with L1, M, 23505.2 ± 3.0, L2, M, 23851.4 ± 3.0, and two minor components L3, M, 26342.4 ± 3.0 and L4, M, 27,425.8 ± 3.0. These subunits (L1–L4) occur with the following relative intensities of 1.0:1.0:0.5:0.5. Finally, a last group with M, over 30,000 contained only one component, D1, M, 371208 ± 0.2. Upon DTT treatment, D1 decreased in relative intensity and two new components appeared with d, M, 15,578.5 ± 0.0, and e, M, 16,149.1 ± 0.8, clearly indicating that D1 was a covalent dimer (data not shown).

The mass spectrum of V2 Hb (Fig. 2C) consisted of two groups of components, one around M, 16,000–18,000 and the other over 30,000. The first group (close-up in Fig. 3A), in
addition to chain b and c, contained five glycosylated isoforms of chain a (a1, Mr 17,636.4 ± 1.0, a2, Mr 17,799.0 ± 1.0; a3, Mr 17,962.3 ± 0.1; a4, Mr 18,451.0 ± 1.1; a5, Mr 18,613.2 ± 0.5) which could be identified by comparison with the mass spectrum of the deglycosylated V2 Hb (Fig. 3). It should be noted that deglycosylation of V1 Hb did not bring any change in the spectrum. The second group contained D1 and another dimer D2, Mr 32,511.7 ± 1.3 (Fig. 2C), which after reduction appeared to be constituted of chains e and f, Mr 16,368.1 ± 0.3 (Fig. 3C). The mass spectrum of C1 Hb (Fig. 2D) resembles that of V2, except that it does not contain dimer D1. Table I lists the average masses of the various components of V1, V2 and C1 Hbs. These results clearly revealed that (i) HBL Hb V1 was devoid of glycoprotein and possessed linker chains (L1–L4), (ii) V2 and C1 Hbs possessed glycosylated chains but no linker chain, and (iii) V2 and C1 were different Hbs, the former containing a dimer absent from the latter.

We have also analyzed the dissociation products V3, V4 and C2 (B). V3 consisted of the dimer D1, while V4 was composed of the monomers b, c and the glycosylated isoforms of chain a. The mass spectrum of C2 was identical to that of C1.

Carbohydrate and Cysteine Residue Contents—Fig. 3 presents the mass spectra of Hb V2 after various treatments. Deglycosylation allowed us to determine the mass of chain a (M, 15,933.4 ± 0.3; Fig. 3B) and we found that the glycosylated isoforms a1–a5 differ in mass by one or more hexose residue(s) (162.1 Da). The difference in mass between chain a5 and chain a (2680.3 Da) is, within experimental error, the mass of the glycan residue (HexNAc)2(Hex)14 (2676.40 Da). Note that ESI-MS cannot distinguish between monosaccharide residues because they have identical masses.

Comparing mass spectra obtained after carbamidomethylation with or without reduction, we have determined the numbers of free Cys residues and disulfide bonds. Fig. 3D shows the results obtained after reduction and carbamidomethylation of V2 Hb. Monomeric chains b and c, and the different glycosylated isoforms of chain a possess one intrachain disulfide bond. Chain b contains also one free Cys residue. Dimers D1 and D2 contain only one interchain disulfide bond which is quite resistant to reduction since ≥50% of the dimers are still present after 60 min with 5 mM DTT (data not shown). Each chain composing D1 and D2 (d, e, and f) contains one intrachain disulfide bond but only chain e has an additional free Cys. We have also found twelve Cys residues on the linker chain L1. Table II summarizes glycan composition and number of Cys residues for all chains.

Quaternary Structure Models of R. pachyptila Hbs—Densitometry analysis of SDS-PAGE profiles for V1 Hb chains AI, AII, AIII, and AIV yielded unclear results and did not allow us to propose a coherent quaternary model for Hb V1 using this technique. However, for V2 Hb, the polypeptide chains BI, BII, BIII, and BIV occur in the ratio 1:1:1:0.5 (average of five gel densitometry analysis) consistent with the following assembly: ((BI, BII, BIII)2BIV)4. In this model containing 28 polypeptide chains, a trimer is composed of a disulfide-bonded dimer of chains BI and BII and a monomeric chain BIII, and two trimers are linked by one chain BIV. A similar model could be applied to C1 Hb.

MaxEnt analysis of ESI-MS spectra produces quantitative relative intensity data. It provides a zero charge spectrum in
which the areas under the peaks are proportional to the sum of the intensities of the peaks in the original raw multicharged spectrum. Using mass measurements of the different Hbs obtained by MALLS (8) and the exact masses of polypeptide chains determined by ESI-MS, we propose coherent models of the quaternary structure of Riftia Hbs. According to these results HBL Hb V1 would consist of 144 globin-like chains (48 monomers and 48 dimers) and 36 linker chains (Table I). In this model, the relative intensities of the four linker chains were L1: L2: L3: L4 = 1:1:0.5:0.5, the globin:linker ratio was 0.75:0.25 and the mass of protein per heme yielded 22,805 g. The primary functional subunit of HBL Hb V1 corresponds to a dodecamer ((cD1)(bD1), with a calculated molecular mass of 200 kDa. This basic subunit should be bound to three linker chains (e.g. 1 L1, 1 L2, and either 1 L3 or L4) corresponding to a total linker mass of 74 kDa. Finally we obtain for the whole molecule a molecular mass of 3285 kDa.

In the same way, Hb V2 (Table IV) and C1 (Table V) would be constituted of 24 globin-like chains providing a calculated mass of 403 and 406 kDa including heme and glycan side chains, respectively. We propose the following molecular models: ((cD1)(bD1)(aD)) for Hb V2 (D being D1 or D2) and ((cD2)(bD2)(aD2)) for Hb C1. The dissociation product V3 is composed of ((D1)3) (99 kDa, calculated mass, Table VI). The dissociation product C2 is composed of ((cD2)(bD2)(aD2)) (206 kDa, calculated mass, Table VII).

**DISCUSSION**

SDS-PAGE has been widely used in the past to study the structure of proteins, including HBL Hbs of annelids (12) and vestimentifera (6, 29). Even though ESI-MS gives more accurate results (see below) very few HBL Hbs have been studied with this technique to date (13–15) and thus we continued using SDS-PAGE for comparative purposes.

Our SDS-PAGE results on Riftia Hbs are in total agreement with those obtained on Lamellibrachia sp. Hbs (6) and clearly reveal that only HBL Hb V1 possesses linker chains with molecular mass of 32 and 36 kDa (AV–AVIII). Moreover, upon reduction these polypeptide chains display bands with a reduced mobility, indicative of even higher molecular masses. This phenomenon has been previously observed in other HBL Hbs (6, 12) and is probably due to the presence of intrachain disulfide bonds (12). It is known that unreduced proteins containing intrachain disulfide bonds possess a more compact hydrodynamic shape in SDS than the fully reduced proteins (30, 31). The primary structure of some linker chains of Lamellibrachia sp. (32), Tylorrhynchus heterochaetus (33), and L. terrestris (34) revealed an important number of cysteine residues which may be able to form intrachain disulfide bonds. In addition, SDS-PAGE overestimates the true molecular mass of

### Table I

| Subunit/chain | Vascular blood | Coelomic fluid | Mean (±S.D.) |
|---------------|---------------|---------------|--------------|
| Massa | V1 | V2 | C1 | |
| a | 16133.3 | 16132.9 | 16134.2 | 16133.5 ± 0.7 |
| b | 16505.6 | 16605.4 | 16806.8 | 16805.9 ± 0.8 |
| c | 16736.4 | 16738.4 | 16737.4 | 16737.4 ± 1.0 |
| d | 16147.0 | 16148.5 | 16148.5 | 16149.1 ± 0.5 |
| e | 16150.2 | 16148.5 | 16148.5 | 16149.1 ± 0.8 |
| f | 16365.7 | 16377.3 | 16377.3 | 16377.3 ± 1.1 |
| D1h | 31720.8 | 31720.5 | 31720.5 | 31720.5 ± 0.3 |
| D2i | 32510.4 | 32512.9 | 32512.9 | 32512.9 ± 0.2 |
| L1 | 23505.2 | 23505.2 | 23505.2 | 23505.2 ± 0.3 |
| L2 | 23851.4 | 23851.4 | 23851.4 | 23851.4 ± 0.4 |
| L3 | 26342.4 | 26342.4 | 26342.4 | 26342.4 ± 0.5 |
| L4 | 27425.8 | 27425.8 | 27425.8 | 27425.8 ± 0.6 |

**a**. Masses (in Da) are for native chains and subunits except for chains d, e, and f which were derived from reduced and carbamidomethylated Hb, hence their masses are with reduced Cys. Estimated error for the single chains a-f is ±1.5 Da; for the glycosylated chains a1–a5 ±2.0 Da; for dimers D1 and D2 and linkers L1–L4, ±3.0 Da.

**b**. Mass corrected for the conversion of Asn to Asp on deglycosylation (42).

**c**. Glycosylated isoforms of chain a.

**d**. Disulfide-bonded dimer d + e. Note that d + e – 6H (for two intra- and one interchain disulfide bonds) is within experimental error of the mass of D1.

**e**. Disulfide-bonded dimer e + f. Note that e + f – 6H = D2 within experimental error.

### Table II

| Chains | Native mass | Red/Cam mass | Number of Cys | Corrected mass |
|--------|-------------|--------------|---------------|----------------|
| a      | 15933.4a   | NDb          | ND            | ND             |
| b      | 16133.5    | 16306.9      | 3             | 16135.7        |
| c      | 16805.9    | 16922.1      | 2             | 16900.0        |
| d      | 17752.7    | 17916.7      | 2             | 17908.0        |
| e      | 18415.0    | NDh          | ND            | ND             |
| f      | 18613.7    | NDh          | ND            | ND             |
| d      | 15757.5    | 15749.7      | 3             | 15757.5        |
| e      | 16147.0    | 16377.3      | 4             | 16149.1        |
| f      | 16365.7    | 16539.3      | 3             | 16368.1        |
| L1     | 23505.2    | 24201.6      | 12            | 23517.0        |

**a**. From partly reduced and carbamidomethylated Hb.

**b**. Mass difference between columns 2 and 3 divided by 57 and rounded off.

**c**. Mass corrected for carbamidomethylation (57.052/Cys). Values are masses with reduced Cys. A mass increase over native mass suggests the existence of an intra-chain disulfide bond.

**d**. Mass difference between chain a and its glycoforms a1–a5.

**e**. Mass calculated for (HexNAc)2(Hex)4.

**f**. Because of the significantly larger differences between the measured and calculated masses for a4 and a5 (4.5 and 3.9 Da, respectively) relative to a1–a3 (0.5, 0.9 and 1.2 Da, respectively) the assignments of the glycan side-chains for the former may be questionable.

**g**. From deglycosylated Hb, corrected for conversion of Asn to Asp on deglycosylation (42).

**h**. ND, not determined.

**i**. From partly reduced Hb (5 mM DTT for 15 min).
components containing glycan or a high content of some amino acids such as proline (35, 36).

In contrast, ESI-MS analysis of Riftia Hbs provides a complete and self-consistent description of their constituent subunits and polypeptide chains, together with mass measurements with an unrivaled accuracy (0.01%). Moreover, the high resolution (~1500) allows polypeptide chains with close molecular masses to be distinguished. Vascular Hb V1 consists of two monomeric globin chains b and c, one disulfide-bonded dimer (D1) of globin chains d and e, and four different linker chains L1–L4. Vascular Hb V2 consists of three monomeric globin chains, a, b, and c, chain a occurring as five glycosylated forms

TABLE III
Polypeptide chain masses and relative intensities from ESI-MS and proposed quaternary model for the HBL Hb V1 of R. pachyptila

| Chains | Massa | BP Ib | S.D.c | Copies using experimental Mf, e | S.D. a | Copies in proposed whole-molecule model | Copies in a one-twelfth model subunit |
|--------|-------|--------|--------|--------------------------------|--------|---------------------------------|---------------------------------|
| b      | 16133.5 | 20.06  | 7.52   | 44                             | 16.33  | 36                             | 3                               |
| c      | 16805.9 | 6.34   | 1.71   | 13                             | 3.56   | 12                             | 1                               |
| d      | 15578.5 | 22.38  | 3.69   | 50                             | 8.30   | 48                             | 4                               |
| e      | 16149.1 | 26.90  | 3.92   | 58                             | 8.50   | 48                             | 4                               |
| L1     | 23505.2 | 8.40   | 0.94   | 13                             | 1.40   | 12                             |                                 |
| L2     | 23851.4 | 7.87   | 1.21   | 12                             | 1.78   | 12                             |                                 |
| L3     | 26342.4 | 3.95   | 0.70   | 5                              | 0.93   | 6                              |                                 |
| L4     | 27425.8 | 4.10   | 0.40   | 5                              | 0.51   | 6                              |                                 |
| Total  | 100    | 200    | 180    | 12                             |        |                                |                                 |

Modelf
(cD1)(bD1)3

a Masses from Table I.
b Percent of base peak intensity from MaxEnt processed ESI-MS spectra.
c Standard deviation for %BPI.
d Number of copies for each chain calculated using the mass of Hb V1 determined by MALLS (8).
e Standard deviation for chain copies.
f Dimer D1 = d + e.

TABLE IV
Polypeptide chain masses and relative intensities from ESI-MS and proposed quaternary model for Hb V2 of R. pachyptila

| Chains | Massa | BP Ib | S.D.c | Copies using experimental Mf, e | S.D. a | Copies in proposed whole-molecule model |
|--------|-------|--------|--------|--------------------------------|--------|---------------------------------|
| b      | 16133.5 | 27.3   | 4.39   | 7                              | 1.18   | 6                               |
| c      | 16805.9 | 4.7    | 0.87   | 1                              | 0.22   | 1                               |
| a1f    | 17637.4 | 5.1    | 1.13   | 1                              | 0.28   | 1                               |
| d      | 15578.5 | 9.5    | 3.26   | 3                              | 0.91   | 4                               |
| e      | 16149.1 | 38.0   | 8.27   | 10                             | 2.22   | 8                               |
| f      | 16368.1 | 15.4   | 8.07   | 4                              | 2.14   | 4                               |
| Total  | 100    | 26     | 6      | 24                             |        |                                 |

Modelg
(cD)(bD)6(a1D)

a Footnotes a to e as in Table III.
f a1 is the most abundant glycosylated isoform of chain a.
g D corresponds to dimer D1 = d + e or D2 = e + f.

TABLE V
Polypeptide chain masses and relative intensities from ESI-MS and proposed quaternary model for Hb C1 of R. pachyptila

| Chains | Massa | BP Ib | S.D.c | Copies using experimental Mf, e | S.D. a | Copies in proposed whole-molecule model |
|--------|-------|--------|--------|--------------------------------|--------|---------------------------------|
| b      | 16133.5 | 27.3   | 1.36   | 6                              | 0.32   | 6                               |
| c      | 16805.9 | 4.32   | 0.19   | 1                              | 0.04   | 1                               |
| a1f    | 17637.4 | 4.93   | 1.17   | 1                              | 0.25   | 1                               |
| e      | 16149.1 | 38.65  | 3.55   | 9                              | 0.84   | 8                               |
| f      | 16368.1 | 24.80  | 5.68   | 6                              | 1.32   | 8                               |
| Total  | 100    | 23     | 24     |                                |        |                                 |

Modelf
(cD2)(bD2)6(a1D2)

a Footnotes a to e as in Table III.
f a1 is the most abundant glycosylated isoform of chain a.
g D2 corresponds to dimer D2 = e + f.
(a1–a5), and two disulfide-bonded dimers, D1 and D2, the latter consisting of chains e and f. These results imply that V2 is not a subunit of V1. Coelomic Hb C1 shares with the vascular Hb V2, the three monomeric globin chains a, b, and c, including the glycosylated isoforms a1–a5, the disulfide-bonded dimer D2 and the absence of linker subunits. However, it lacks the dimer D1 and therefore, in contrast with previous studies (16), our results show that V2 and C1 are different Hbs.

Examining our results with those obtained with this technique on the earthworm, Lumbricus (13), and the leech Macrobdella (14) HBL Hbs, it is clear that all three Hbs are built up from the same kind of constituents, e.g., monomeric globin chains (~16 kDa), linker chains (~26 kDa), and trimers or dimers of globin chains (~53 or 32 kDa, respectively). In Riftia, the linker chains L1–L4 are only found in V1 Hb, in agreement with the idea that they are required for the maintenance of the HBL structure by analogy with annelid HBL Hbs, such as earthworm Lumbricus (13) and the leech Macrobledella (37), and annelid chlorocruorin, such as the polychaete Eudistylia (38). Riftia and Lamellibrachia (6) HBL Hbs most closely resemble that of the achaete Macrobledella Hb (14). These Hbs contain disulfide-bonded dimers whereas oligochaetes and polychaetes Hbs usually contain disulfide-bonded trimers (12). However, in contrast with annelid species, the vestimentifera Riftia pachyptila and Lamellibrachia sp. (29) possess an original multi-hemoglobin system with two extracellular Hbs (V2 and C1) in addition to the HBL V1.

ESI-MS also offers reliable information concerning the number of Cys residues and disulfide bonds in native proteins, by comparing data obtained from native or reduced forms with their carboxymethylated counterparts (see e.g. Fig. 3, C and D). All annelid and tube worm globin-like chains known to date can be classified into four groups. All groups possess an intrachain disulfide bond, and three of these groups contain a supplementary free Cys, at different positions, participating in an interchain disulfide bond (29, 39). In Riftia Hbs (Table II), all chains have at least two Cys, forming an intrachain bond. Chains d, e, and f have an additional Cys forming an interchain bond, but only chains b and e have a free Cys residue. We have been able to analyze only one linker chain (L1) and we found a considerable number of Cys residues, all participating in intrachain bonds. The knowledge of Cys contents is not only important for structural implications, but, in the case of vestimentifera, it may also have a functional meaning since previous studies have suggested a role for Cys in the unique ability of these Hbs to reversibly bind sulfide (6, 16, 32).

Using the precise masses of native Riftia Hbs (8) and the present results on polypeptide chain composition, we have been able to propose coherent models of the quaternary structure of these heteromultimeric proteins. Data derived from SDS-PAGE analysis of V2 and C1 Hbs yields a 28-chain model in total agreement with the model proposed for Lamellibrachia sp. (6). Using data derived from ESI-MS results we found a slightly different model composed of 24 globin chains, suggesting an association of 8 trimeric subunits each composed of one disulfide-bonded dimer and a monomeric chain. The total calculated mass of this model, including heme groups and glycans side chains, is very close to the experimental mass (see Tables IV and V).

The model found for V1 Hb (Table III) consists of 144 globin chains and 36 linker chains, providing a total calculated mass of 3285 kDa, including heme groups. This is very close to the experimental data obtained by STEM mass mapping (3396 ± 540 × 103) and MALLS (3503 ± 13 × 103) (8). In addition, this model yields a heme content consistent with experimental data found previously (16). The primary functional subunit of HBL Hb V1 corresponds to a dodecamer with a molecular mass of 200 kDa and should be linked with three linker chains corresponding to a mass of 74 kDa. This assemblage provides a molecular mass of 274 kDa, in good agreement with the one-
twelfth mass of 283 kDa determined by STEM mass mapping (8). As for other HBL Hbs analyzed with ESI-MS to date (13–15), our data for Riftia HBL Hb V1 fit well with the “bracelet model” proposed for L. terrestris (40). This model has recently been substantiated by three-dimensional reconstruction based on cryomicroscopy analysis (41).

The results obtained with Riftia Hbs illustrates the power and scope of ESI-MS in the analysis of large, heteromultimeric protein complexes. The precise knowledge of the quaternary and scope of ESI-MS in the analysis of large, heteromultimeric protein complexes. The precise knowledge of the quaternary...