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An electrospray ionization mass spectrometric study of the giant, extracellular, hexagonal bilayer hemoglobin of the leech *Haemopis grandis* provides a complete enumeration of its subunits

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Abstract

The complex, multiply charged electrospray ionization mass spectra of the extracellular, ~3500 kDa, hexagonal bilayer hemoglobin from the leech *Haemopis grandis* and its carbamidomethylated, reduced and reduced/carbamidomethylated forms were deconvoluted using a maximum entropy approach to provide the corresponding zero-charge spectra. Three groups of peaks were observed: monomeric globin chains a1–a4 at ~17 kDa (16 634.1, 17 013.4, 17 592.9, and 17 573.3 Da with relative intensities of 16:4:12:1, respectively), linker chains L1–L3 at ~24 kDa (24 004.2, 24 449.2 and 24 548.3 Da, with relative intensities of 5:1:2.5, respectively) and subunits D1 and D2 at 32 501.1 and 32 629.6 Da, respectively, with equal intensities. Reduction of the native hemoglobin with dithiothreitol resulted in a decrease in the mass of linker L2 by 115.1 Da, indicating cysteinylation, the disappearance of subunits D1 and D2 and the concomitant appearance of globin chains b (16 098.8 Da), c1 (16 403.9 Da), and c2 (16 532.5 Da), suggesting that subunits D1 and D2 are disulfide-bonded dimers b + c1 and b + c2, respectively. All the globin chains appear to have one intrachain disulfide bond, and globins b, c1, and c2 have an additional Cys which forms the interchain disulfide bond in D1 and D2. The linkers L1–L3 contain 10, 9, and 10 Cys residues, respectively, all forming intrachain disulfide bonds, except for the odd residue in L2 which is proposed to be the site of cysteinylation. The relative intensities of the three groups of peaks a1 + a2 + a3 + a4:L1 + L2 + L3:D1 + D2 are 1.65:1.7:0.8 in native hemoglobin. All the subunits in a second sample evinced substantial glycosylation, with up to five hexoses per subunit. A model of the quaternary structure of *Haemopis* hemoglobin is proposed, consisting of 72 monomeric globins and 36 globin dimers for a total of 144 globin chains and 42 linkers with a calculated total mass of 3506 kDa. (Int J Mass Spectrom 188 (1999) 105–112) © 1999 Elsevier Science B.V.

Keywords: Electrospray ionization mass spectrometry; Leech; Hemoglobin; Subunits

1. Introduction

The broad variety in the structure and function of the extracellular Hb of invertebrates stands in surprising contrast to the relative uniformity of the vertebrate Hbs which are generally intracellular and tetrameric. The former range from single-chain globins to very large and complicated structures consisting of 200 and more globin chains. Within this panorama of structures, the extracellular, hexagonal bilayer (HBL) Hbs of annelids and vestimentiferans form one of four recognizable classes of quaternary structure [1]. They have a sedimentation constant of ~60 S, a mass of about 3600 kDa, an acidic isoelectric point and a characteristic, hexagonally symmetric electron microscope image [2–7]. Recent work has demonstrated

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that they consist of two types of chains: heme-containing, ~17 kDa chains whose amino acid sequences place them firmly within the globin family and the heme-deficient, 24–32 kDa linker chains which are unrelated to globins, constitute about a third of the native complex and are responsible for the formation of the HBL structure [7]. A common model of quaternary structure appears to explain satisfactorily the known facts: 12, ~200 kDa complexes of heme-containing globin chains, i.e. 144, ~17 kDa chains, linked together by 30–40 heme-deficient, linker chains [7–9].

The leeches represent one of the three classes of annelids, in addition to terrestrial oligochaetes and marine polychaetes. Early studies of annelid extracellular hemoglobins using polyacrylamide gel electrophoresis in the presence of SDS showed that there were differences in their constituent subunits: although most Hbs consisted of monomeric globin subunits and subunits of about 24–28 kDa which could not be reduced, the leech Hb differed from the oligochaete and polychaete Hbs in having an ~30 kDa subunit which could be reduced to monomeric subunits, instead of a 50 kDa subunit which could be reduced to three monomeric subunits [10–12]. We describe here the results of an electrospray ionization mass spectrometric study of the Hb from the leech *Haemopis grandis*, which provides a detailed description of the subunits and polypeptide chains comprising this giant globin complex.

2. Materials and methods

2.1. Materials

The Hb was isolated from two batches of live leeches obtained from W. A. Lemberger Co. (Oshkosh, WI 54903) and St. Croix Biological (Stillwater, MN 55082) as described elsewhere [13], except that homogenization of the leeches was carried out in the presence of a protease inhibitor cocktail (#1697-498, Biochemical Div., Boehringer Mannheim Corp., Indianapolis, IN 46250). The concentration of the Hb was determined at 280 nm using the absorptivity of 2.19 ± 0.08 mL mg^{-1} cm^{-1} [14]. Hb was reduced with DTT in the presence of 6 M guanidine hydrochloride and carbamidomethylated with iodoacetamide [15].

2.2. Preparation of samples for ESI-MS

A stock solution of Hb was made in water or 0.05%–0.3% aqueous formic acid to give a concentration of 5 μg/μL. Working solutions of native Hb, actually denatured native Hb, were 0.25–0.5 μg/μL in 50% aqueous acetonitrile containing 0.2% formic acid. Carbamidomethylated Hb was prepared by mixing 10 μL of Hb stock solution, 2 μL of 1 M aqueous ammonium bicarbonate, 2.5 μL of 0.2 M aqueous iodoacetamide solution and 20 μL water, allowing to stand for 10 min, then adding 20 μL of 1.0% formic acid and 50 μL acetonitrile to make the solution for ESI-MS analysis. Reduction was effected by adding 10 μL of 0.1 M DTT to 50 μL aliquot of the Hb stock solution mixed with 30 μL water and 10 μL aqueous 1 M ammonium bicarbonate to bring the pH to 8–9. After mixing, reduction was allowed to proceed at room temperature. At suitable time intervals (1, 5, and 10 min) two 10 μL aliquots were removed. To one, 20 μL of 1.0% formic acid, 20 μL water and 50 μL acetonitrile were added to provide a working solution of reduced Hb. To the other aliquot, 2.5 μL of 0.2 M iodoacetamide and 20 μL water were added, mixed and allowed to stand at room temperature for 10 min. Then 20 μL of 1% formic acid and 50 μL acetonitrile were added to make a working solution for ESI-MS analysis of reduced, carbamidomethylated Hb.

2.3. Electrospray ionization mass spectrometry

The data were acquired on a Quattro II electrospray mass spectrometer (Micromass UK Ltd., Altrincham, Cheshire, UK), using sample concentrations of 0.5 μg/μL in 50/50 acetonitrile/water containing 0.2% formic acid. The sample flow rate into the electrospray source was 5 μL/min and data were typically acquired over 3 min. ESI-MS produces a series of multiply charged ions on a mass-to-charge ratio (m/z) scale from each protein in the sample. On

this scale, $m/z = (M_r + nH)/n$, where M_r is the mass of the protein, H is the mass of the proton, and n is an integer in a series of consecutive integers. Since leech Hb consists of several protein components each producing 5–10 multiply charged ions in a series, the acquired spectra were processed in order to condense each series into a single peak on a true molecular mass scale. Processing used a maximum entropy (MaxEnt) based approach [16,17] employing the MemSys5 program (MaxEnt Solutions Ltd., Cambridge, UK) incorporated as part of the Micromass MassLynx software suite. Mass scale calibration employed the multiply charged series from horse heart myoglobin (Sigma Chemical Co., St. Louis, MO 63178), 16 951.5 Da [18]. Molecular weights are based on the following atomic weights of the elements: C = 12.011, H = 1.007 94, N = 14.006 74, O = 15.9994, and S = 32.066 [19].

3. Results

Figs. 1 and 2 show the ESI-MS results obtained with the Hb from the first batch of live *Haemopis*. Figure 1 shows (A) the original mass-to-charge spectrum of denatured Hb, (B) the mass-to-charge spectrum of the denatured Hb after deconvolution by the MaxEnt program, (C) the MaxEnt deconvoluted spectrum of the Hb reduced for 5 min with 10 mM DTT and (D), the MaxEnt deconvoluted spectrum of the reduced/carbamidomethylated Hb. The spectrum of the denatured Hb [Fig. 1(B)] clearly reveals three groups of components at around 17, 24, and 33 kDa. The 17 and 24 kDa regions are shown on expanded scales in Fig. 2(A)–(C) and in the insets to Fig. 1(B)–(D), respectively. Table 1 lists the mean masses of the components observed in the three mass regions.

The globin group of peaks [Fig. 2(A)], consists of four components, a1 (16 634.1 Da), a2 (17 013.4 Da), a3 (17 592.9 Da), and a4 (17 573.3 Da), with the following relative intensities: a1:a2:a3:a4 = 16:4:12:1 (Table 1).

The linker group of peaks [Fig. 1(B)] comprises three peaks, L1 (24 004.2 Da), L2 (24 449.2 Da), and L3 (24 548.3 Da) with relative intensities of 5:1:2.5, respectively (Table 1).

The third group of peaks at ~33 kDa [Fig. 1(B)] consists of peaks D1 (32 501.1 Da) and D2 (32 629.6 Da) of approximately equal intensities.

Treatment of the native Hb with 5 mM DTT for 5–30 min leads to the disappearance of the ~33 kDa peaks and to the concomitant appearance of three new peaks in the 16 kDa region [Figs. 1(C) and 2(B)]: b (16 098.8 ± 0.3 Da), c1 (16 403.9 ± 0.4 Da), and c2 (16 532.5 ± 0.1 Da). The dissociation of the D1 and D2 peaks upon treatment with DTT, suggests that they are disulfide-bonded dimers of globin chains b + c1 and b + c2, respectively. Reduction also decreases the mass of linker chain L2 by 115.1 Da [Fig. 1(C)], implying that it is cysteinylated. The presence of a peak at 24 737.3 Da in the spectrum of reduced and carbamidomethylated Hb [Fig. 1(D) inset] is probably due to incomplete reduction of L2.

Figure 3 shows the MaxEnt processed ESI zero-charge spectra of the Hb obtained from the second batch of *Haemopis*. The complete spectrum [Fig. 3(A)] shows a more complicated fine structure pattern in each of the three groups of peaks than was observed from the first batch. The three regions presented using an expanded mass scale [Fig. 3(B)–(D)] show multiple additions of 162.1 Da (±1.1 Da, $n = 22$) to the subunits observed from the first batch, suggesting that this fine structure is due to extensive glycosylation of practically all the observed subunits, with up to five hexoses per subunit. Fig. 3(B) shows that all four globin chains exhibit adducts with one and two hexose groups. Fig. 3(C) demonstrates that L1 forms adducts with up to five hexoses, while L2 and L3 form adducts with up to two and three hexoses, respectively. The disulfide-bonded dimers D1 and D2 appear to form adducts with up to three and four hexoses, respectively [Fig. 3(D)].

4. Discussion

The results presented in Sec. 3 illustrate our general approach to the complete characterization of the polypeptide chains and disulfide-bonded subunits comprising the giant, cysteine-rich, HBL Hbs: the Hbs of the leech *Macrobdella* [20], the earthworm

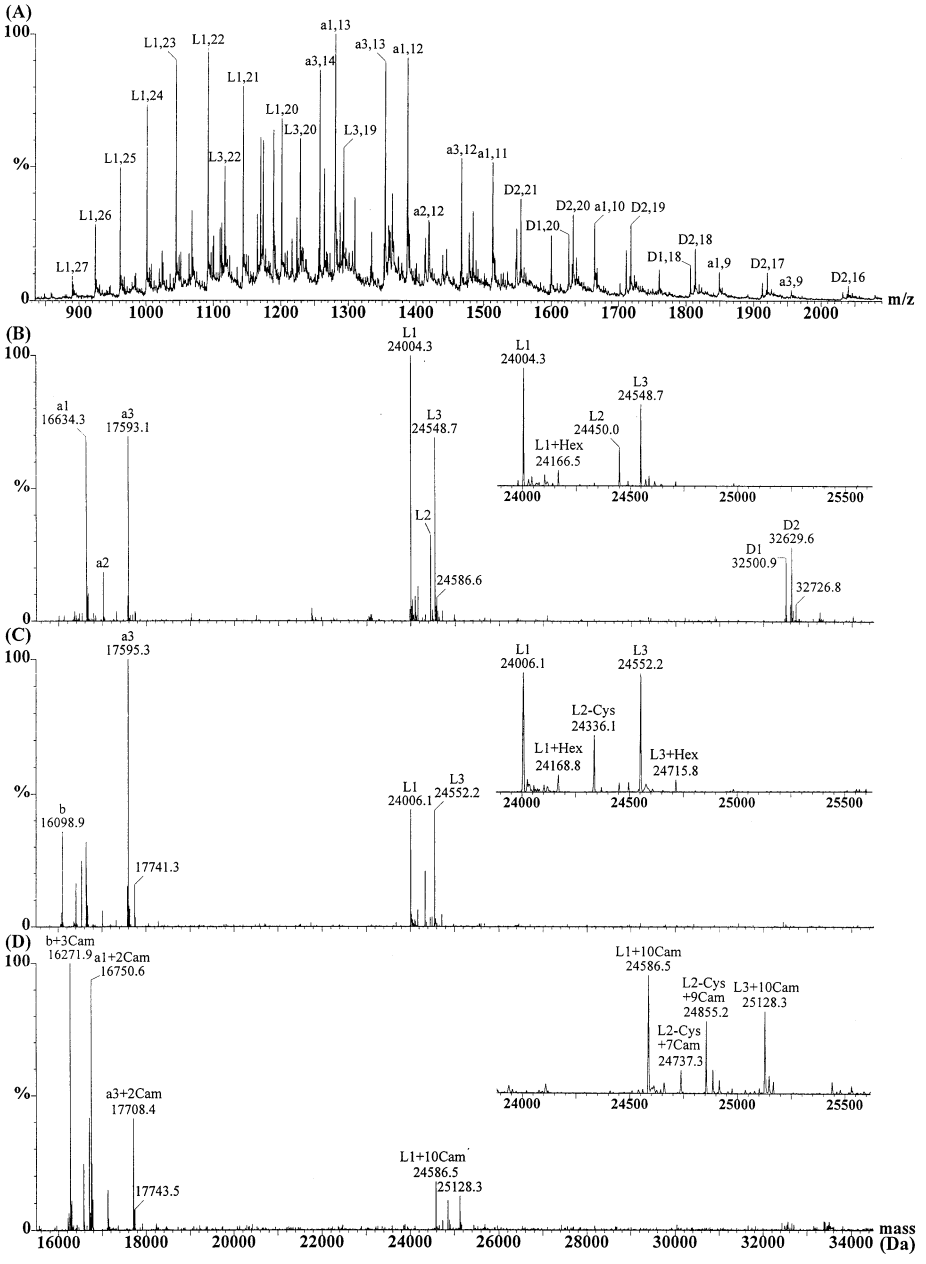

Fig. 1. (A) The original ESI mass-to-charge spectrum from the first batch of denatured *Haemopis* Hb. (B) The data shown in (A) after deconvolution by the MaxEnt program. (C) The MaxEnt processed spectrum of the Hb after reduction for 5 min with 10 mM DTT. (D) The MaxEnt spectrum of the reduced and carbamidomethylated Hb. The insets show the linker region on an expanded mass scale. In (A), the integer after the component identifier refers to the number of protons on the molecule, e.g. a1, 12 = component a1 with 12 protons. Cam = carbamidomethylated cysteine.

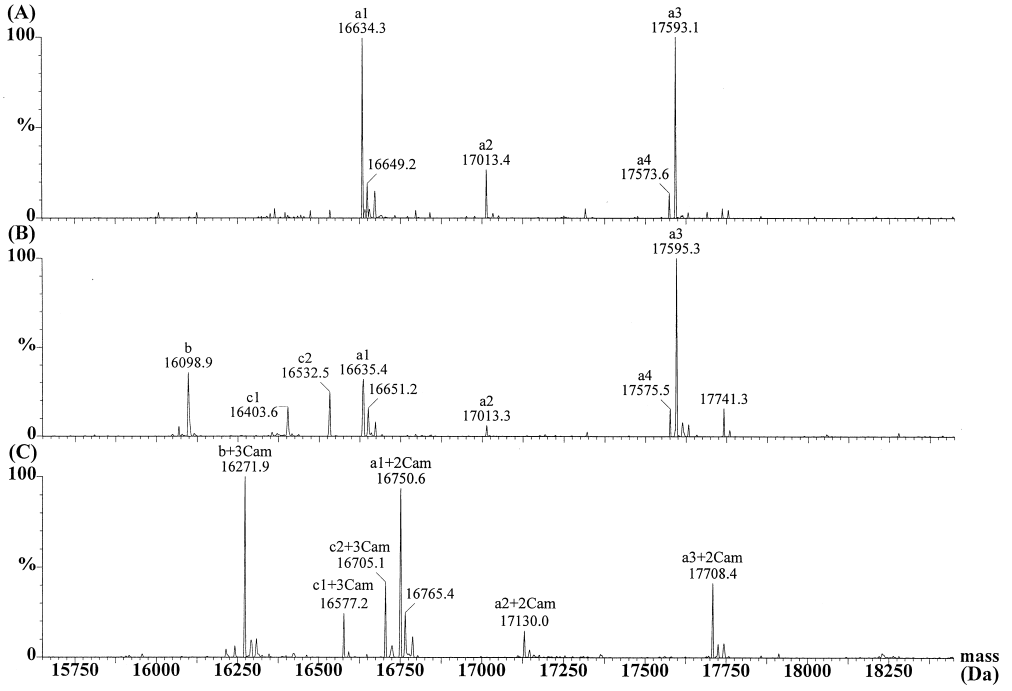

Fig. 2. The globin region of the spectra given in Fig. 1(B)–(D) shown on an expanded mass scale: (A) the denatured Hb, (B) the reduced Hb and (C), the reduced and carbamidomethylated Hb.

Table 1
Masses from MaxEnt processing of ESI-MS of native *Haemopis* Hb[a]

Chain/subunit	Native[b]	Rel. int[c]	Chain	Reduced[d]	Red/cam[e]	Corrected[f]	No. Cys[g]
a1	16 634.1 ± 0.5	0.8	a1	16 635.0 ± 0.4	16 750.7 ± 0.4	16 636.6	2
a2	17 013.4 ± 0.5	0.2	a2	17 013.2 ± 0.7	17 130.0 ± 1.2	17 015.9	2
a3	17 592.9 ± 0.5	0.6	a3	17 594.6 ± 0.5	17 708.7 ± 0.3	17 594.6	2
a4	17 573.3 ± 0.8	0.05	a4	17 575.6 ± 0.7	ND	ND	ND
L1	24 004.2 ± 0.4	1.0	L1	24 005.5 ± 0.6	24 585.2 ± 1.5	24 014.7	10
L2	24 449.2 ± 1.1	0.2	L2-Cys	24 334.1 ± 1.9	24 853.4 ± 2.0	24 339.9	9
L3	24 548.3 ± 0.6	0.5	L3	24 551.1 ± 0.7	25 127.9 ± 1.3	24 557.4	10
D1	32 501.1 ± 0.7	0.4	b	16 098.8 ± 0.3	16 271.6 ± 0.5	16 100.4	3
D2	32 629.6 ± 0.8	0.4	c1	16 403.9 ± 0.4	16 577.0 ± 0.4	16 405.8	3
			c2	16 532.5 ± 0.1	16 705.2 ± 0.5	16 534.0	3

[a] Masses in Da; estimated errors are ±1.0 Da for a1–a4, b, c1, and c2, ±2.0 Da for L1–L3 and ±3.0 Da for D1 and D2; ND = not determined.

[b] Mean of six determinations ±SD.

[c] Relative intensity for native Hb.

[d] Mean of four determinations ±SD with partially reduced Hb.

[e] Mean of four–nine determinations ±SD with reduced/carbamidomethylated Hb.

[f] Corrected for carbamidomethylation (57.052/Cys). Values are masses with Cys reduced.

[g] No. of Cys = (red/cam mass − reduced mass)/57.052 rounded to nearest integer. No free Cys were detected in the native Hb.

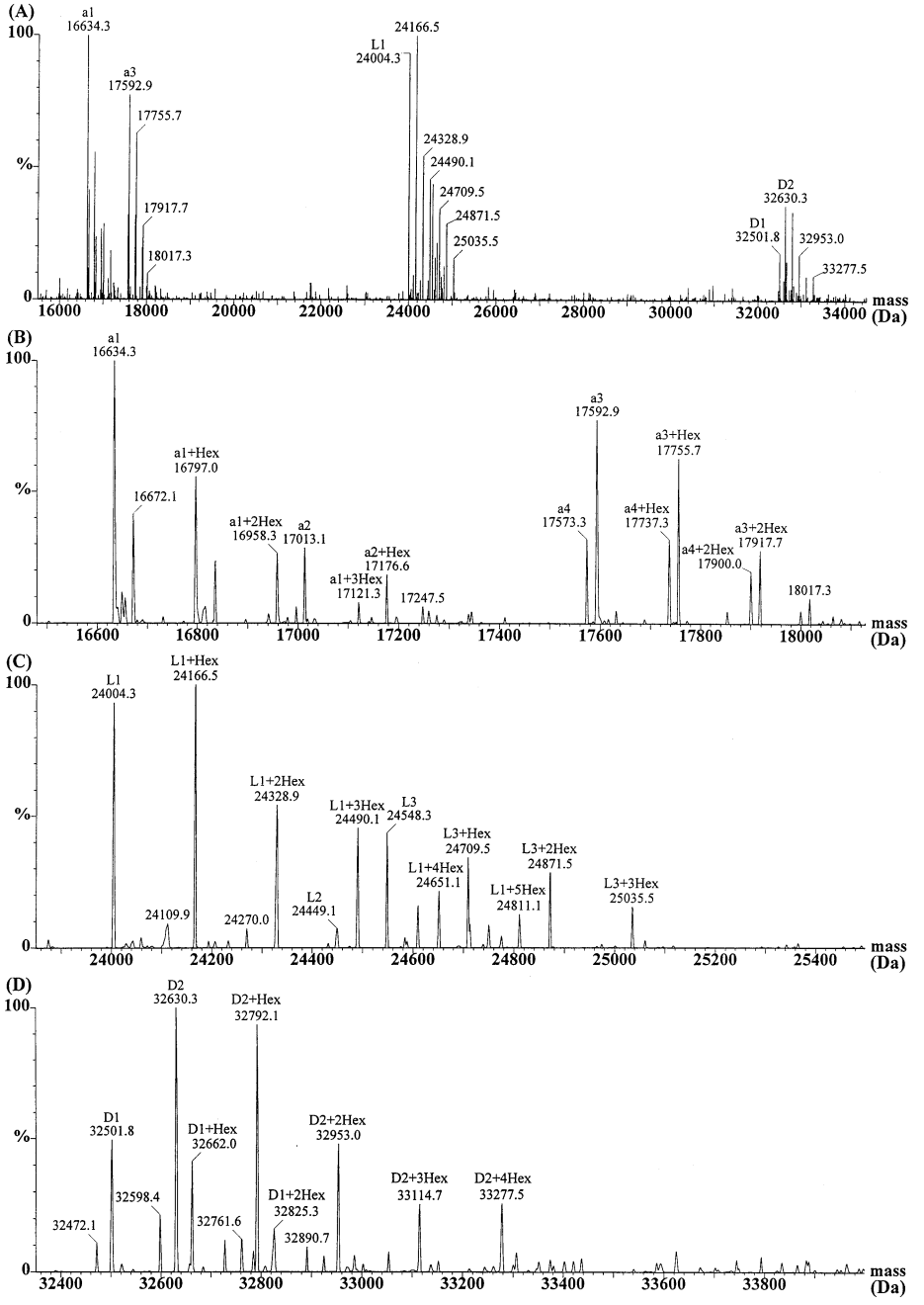

Fig. 3. MaxEnt processed ESI mass spectra of the second batch of native *Haemopis* Hb. (A) Survey spectrum and expanded mass scale spectra of the globin region (B), the linker region (C), and the dimer region (D), demonstrating the extensive glycosylation of the constituent subunits and polypeptide chains. Hex = hexose.

Lumbricus [21], the vestimentiferan *Riftia* [22], the polychaetes *Tylorrhynchus* and *Arenicola* [23,24] and the chlorocruorin of the polychaete *Eudistylia* [25]. This approach consists of four steps. First, the native Hb is analyzed in denaturing solvent in order to determine the masses of the components that exist as covalently bound entities, i.e. as linker chains and disulfide-bonded multimeric subunits. Second, it is reduced with DTT in order to determine the approximate masses of the chains that compose the multimeric subunits. Third, the native Hb is carbamidomethylated in order to determine the number of free Cys residues associated with each subunit. Finally, the Hb is reduced and carbamidomethylated in order to determine the total number of Cys present in each globin and linker chain and the precise reduced masses of each chain. The number of Cys residues present in each subunit or polypeptide chain is calculated from the difference between the reduced and reduced/carbamidomethylated masses. This approach provides a complete enumeration of all the constituent disulfide-bonded subunits and chains, their masses as well as the number of free Cys and disulfide-bonded cysteine residues for each subunit and polypeptide chain. By using MaxEnt, all this information is produced from the Hb and its derivatives without separating the components prior to analysis.

Table 1 shows, in addition to the masses of the subunits and polypeptide chains comprising *Haemopis* Hb, the number of cysteines present in all the globin and linker chains with the exception of globin a4. Although no free Cys was detected in the native Hb, it appears that all the cysteine residues participate in intra- and intermolecular disulfide bonds. Table 2 presents the masses of all the chains (except a4) and subunits calculated on that basis. The globin chains a1–a3 all appear to have one intramolecular disulfide bond. The linker subunits L1 and L3 have five intramolecular disulfide bonds and L2 only four, with the odd cysteine residue being cysteinylated. Overall the self-consistency of the masses is very good and well within experimental errors. The presence of a peak at 24 737.3 Da in the spectrum of reduced/carbamidomethylated Hb [Fig. 1(D), inset] suggests that one of the four intrachain disulfide bonds in linker L2 is more resistant to reduction than the other three.

Table 2
Self-consistency of mass assignments[a]

Chain/subunit	No. disulfide bonds	Calculated mass[b]	Mass diff[c]
a1–2H	1	16 634.6	0.5
a2–2H	1	17 013.9	0.5
a3–2H	1	17 592.6	−0.3
L1–10H	5	24 004.6	0.4
L2–8H + Cys	5[d]	24 451.0	1.8
L3–10H	5	24 547.3	−1.0
D1 = b + c1–6H	3	32 500.2	−0.9
D2 = b + c2–6H	3	32 628.4	−1.2

[a] Masses in Da.
[b] Calculated from corrected mass in Table 1.
[c] Between native mass and calculated mass.
[d] Comprising four intrachain disulfide bonds and one due to cysteinylation of the odd cysteine.

We have no explanation for the putative glycosylation in the second sample of *Haemopis* Hb. The glycans in this case appear to be multiple addition of single hexose units, possibly N-glycosyl additions to lysines and the N-terminus, somewhat akin to glycation in human Hb but at a substantially higher level. They are quite different from those observed in linker L1 and globin a in *Lumbricus* Hb [21] and in globin a of *Riftia* Hb [22], which consist of $HexNAc_2Hex_n$, where $n = 6–9$ in *Lumbricus* Hb and 8–10 and 13–14 in *Riftia* Hb. In contrast to *Haemopis* Hb, the glycosylation in *Lumbricus* Hb is similar from sample to sample, suggesting perhaps some variable environmental cause in the case of the leech such as the season or their food. The only other case where we have observed similar sample dependent glycosylation to that observed in *Haemopis* Hb is with another leech *Nephelopsis* (unpublished observations).

The availability of accurate masses for the constituent polypeptide chains and subunits of *Haemopis* Hb permits a reliable model of its quaternary structure to be proposed. The ESI-MS results provide no clue as to the amino acid sequence similarity between the six globin chains on one hand and the three nonglobin, linker chains, on the other. The basic subunits are thus a monomer globin of 17.06 kDa, a disulfide-bonded dimer of 32.56 kDa and linker subunit of 24.22 kDa,

the masses weighted according to the relative intensities in column 3 of Table 1. A common model of the quaternary structure of hexagonal bilayer hemoglobins, consisting of 12 × 12 globin chains and 36 or 42 linker chains, has been found to provide good agreement with the experimentally determined masses [7]. In the specific case of *Haemopis* Hb, the globin portion of this giant complex is comprised of monomer globin chains and disulfide-bonded dimers in the ratio of 2:1 according to the ESI-MS data [a1 + a2 + a3 + a4:D1 + D2 = 1.65:0.8 (column 3, Table 1)]: 72 monomers (1228 kDa) and 36 dimers (1172 kDa). Together with 42 linkers (872 kDa), the total calculated mass including 144 heme groups is 3417 + 89 = 3506 kDa (3361 kDa with 36 linkers). The calculated mass is close to the masses obtained by sedimentation equilibrium for other leech Hbs, 3710 kDa for *Haemopis sanguisuga* [12] and 3540 kDa for *Macrobdella decora* [20] Hbs and the 3560 kDa determined for *Macrobdella* Hb by STEM mass mapping [14]. It should be pointed out that recent three-dimensional reconstructions using electron cryomicroscopy of *Macrobdella*, *Riftia* and *Lumbricus* Hbs [26–29] and of *Eudistylia* chlorocruorin [30], have provided strong support for such a model.

Abbreviations

Hb—hemoglobin; HBL—hexagonal bilayer; SDS—sodium dodecyl sulfate; ESI-MS—electrospray ionization mass spectrometry; EDTA—ethylenediamine tetraacetic acid; DTT—dithiothreitol; STEM—scanning transmission electron microscopy.

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