Electrospray Ionization Mass Spectrometric Determination of the Molecular Mass of the ~200-kDa Globin Dodecamer Subassemblies in Hexagonal Bilayer Hemoglobins*

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Hexagonal bilayer hemoglobins (Hbs) are ~3.6-MDa complexes of ~17-kDa globin chains and 24–32-kDa, nonglobin linker chains in a ~2:1 mass ratio found in anelids and related species. Studies of the dissociation and reassembly of Lumbricus terrestris Hb have provided ample evidence for the presence of a ~200-kDa linker-free subassembly consisting of monomer (M) and disulfide-bonded trimer (T) subunits. Electrospray ionization mass spectrometry (ESI-MS) of the subassemblies obtained by gel filtration of partially dissociated L. terrestris and Arenicola marina Hbs showed the presence of noncovalent complexes of M and T subunits with masses in the 213.3–215.4 and 204.6–205.6 kDa ranges, respectively. The observed mass of the L. terrestris subassembly decreased linearly with an increase in de-clustering voltage from ~215,400 Da at 60 V to ~213,300 Da at 200 V. In contrast, the mass of the A. marina complex decreased linearly from 60 to 120 V and reached an asymptote at ~204,600 Da (180–200 V). The decrease in mass was probably due to the progressive removal of complexed water and alkali metal cations. ESI-MS at an acidic pH showed both subassemblies to consist of only M and T subunits, and the experimental masses demonstrated them to have the composition M6T6. Because there are three isomers of M and four isomers of T in Lumbricus and two isomers of M and 5 isomers of T in Arenicola, the masses of the M6T6 subassemblies are not unique. A random assembly model was used to calculate the mass distributions of the subassemblies, using the known ESI-MS masses and relative intensities of the M and T subunit isomers. The expected mass of randomly assembled subassemblies was 213,436 Da for Lumbricus Hb and 204,342 Da for Arenicola Hb, in good agreement with the experimental values.

The giant extracellular HBL1 Hbs found in most terrestrial, aquatic, and marine annelids and in deep sea anelids and vestimentiferans are ~3.6-MDa complexes of globin subunits and nonglobin, linker chains and represent a summit of complexity for oxygen-binding heme proteins (1–4). The most extensively studied complex is the Hb from the common earthworm Lumbricus terrestris. Based on the finding of a ~200-kDa globin subassembly upon mild, partial dissociation of the Hb at neutral pH, a “bracelet” model of its quaternary structure was proposed to consist of twelve ~200-kDa globin subassemblies attached to a central scaffolding of 36–42 linker chains (24–32 kDa) (5). Scanning transmission electron microscopy mass mapping of the isolated globin subassembly showed it to have a mass of 202 ± 26 kDa, consonant with it being a dodecamer of globin chains (~17 × 12 = 204 kDa), [d1],[bac]3 consisting of three copies each of the monomer M (chain d) and the disulfide-bonded trimer T (chains b + a + c) (6); in addition, this subassembly was found to be an obligate intermediate in the dissociation and reassembly of the HBL structure (7). A complete ESI-MS determination of the masses of the constituent globin and linker chains and the disulfide-bonded trimer subunit of Lumbricus Hb provided a calculated mass of 213.434 kDa for the subassembly [M6][T6] (8). Furthermore, the calculated masses for the Hb comprised of 12 subassemblies and 36 or 42 linker chains, 3.523 and 3.687 MDa, respectively, were in good agreement with the masses determined by scanning transmission electron microscopy and sedimentation equilibrium (3.56 ± 0.13 and 3.41 ± 0.39 MDa, respectively) (8). ESI-MS studies of several other HBL Hbs by Green and his collaborators (9–15) have provided accurate masses for all the constituent subunits as well as their relative proportions. The masses calculated based on the model proposed for Lumbricus Hb are in satisfactory agreement with the experimentally determined masses of the native Hbs. Furthermore, three-dimensional reconstructions of several HBL Hbs using cryoelectron microscopy have demonstrated unequivocally the overall correctness of the bracelet model (16–21). Concurrent studies of Lumbricus Hb and its reassembly subsequent to dissociation at an alkaline pH by A. F. Riggs and his group have yielded quite different results (22–24). Multiple angle laser light scattering was used to determine the mass of the Hb, 4.1 ± 0.1 MDa, and reassembly of the isolated M and T subunits was interpreted to indicate a hexadecamer subassembly [M6][T16] ⌡ ⌡ ⌡ ⌡ 285 kDa (22–24). Based on these results, a Hb model was proposed consisting of 12 hexadecamer subassemblies and 24 linker chains. A key point in resolving the differences is a reliable mass for the globin subassembly. We present the results of an ESI-MS study that provides accurate masses for the globin subassemblies isolated from the HBL Hbs of L. terrestris and the marine polychaete Arenicola marina.

EXPERIMENTAL PROCEDURES

Materials—L. terrestris Hb was prepared as described previously (25). A. marina blood was obtained by direct puncture of the ventral vessel of live animals collected near Roscoff, and the Hb was prepared

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1 The abbreviations used are: HBL, hexagonal bilayer; M, monomer subunit; T, disulfide-bonded trimer subunit; ESI, electrospray ionization; ESI-MS, electrospray ionization mass spectrometry.

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as described elsewhere (12). The Hbs were dissociated at a neutral pH in the presence of ~4 M urea and subjected to low-pressure, isocratic gel filtration at room temperature (20 ± 2 °C) using a fast protein liquid chromatography system (Amersham Pharmacia Biotech) and 2.5 × 50-cm columns of Superox S12 or S6 (Amersham Pharmacia Biotech). The flow rate was 0.5 ml/min and the absorbance of the eluate was monitored at 280 nm. The concentrations of the Hb and the globin subassembly were determined from the absorbance of the cyanmet forms at 540 nm using the extinction coefficients 0.442 and 0.656 ml/mg · cm⁻¹, respectively (12, 26).

**Electrospray Ionization Mass Spectrometry—** Sample solutions in 10 mM ammonium acetate were introduced at 4 μl/min into the ESI source of either a Quattro LCZ, a tandem quadrupole instrument whose m/z range was specially extended to 8000, or a quadrupole time-of-flight instrument (Q-Tof) (Micromass UK Ltd., Wythenshawe, Manchester, United Kingdom) with a m/z range of 14,000. Data were accumulated over 5–10 min. Mass scale calibration used the Cs (n= 1, m/z 204.0) and ammonium acetate was added to a concentration of 10 mM.

**RESULTS**

**ESI MS of Dodecamer Globin Subassembly—** Fig. 1 shows the ESI m/z spectrum (pH 7) of the ~200-kDa globin subassembly isolated from Lumbricus Hb by gel filtration of the partially dissociated Hb as performed repeatedly in our earlier studies (5–7). It was obtained on the quadrupole time-of-flight instrument at a de-clustering voltage of 145 V. Three sets of peaks were observed: (a) one set of peaks corresponding to the intact subassembly with charge states ranging from 29+ to 38+, with the 34+ and 35+ states being the most abundant; and (b) two other sets of peaks corresponding to the two subunits comprising the subassembly, i.e. the ~55-kDa trimer T with charge states ranging from 14+ to 18+ and the ~17-kDa monomer M with charge states ranging from 6+ to 9+, respectively. The inset in Fig. 1 shows the result of deconvoluting the part of the spectrum containing the multiply charged subassembly peaks by MaxEnt: a single peak at a mass of 213,909 Da is observed in the 100–330 kDa range.

**ESI-MS of Arenicola Hb Subassembly—** The gel filtration elution profile of partially dissociated Arenicola Hb at a neutral pH was very similar to the elution profile obtained with Lumbricus Hb by a variety of means (5–7) and also exhibited a ~200-kDa subassembly peak. Fig. 2A presents the ESI m/z spectrum of the Arenicola Hb subassembly obtained at neutral pH on the extended m/z range quadrupole instrument. The noncovalent complex is observed with charge states similar to those of Lumbricus (Fig. 1), but it is the dominant species in this case, with almost insignificant levels of M and T, suggesting that the Arenicola subassembly is more stable under ESI-MS conditions than that of Lumbricus. The inset in Fig. 2A shows the result of deconvoluting the raw m/z data by MaxEnt and exhibits a principal peak at 204,753 Da with a much smaller subsidiary peak at 205,308 Da. Fig. 2B shows the ESI m/z spectrum of the Arenicola Hb subassembly obtained under the same mass spectrometer conditions as described for Fig. 2A but with the pH value reduced to pH 3.5. Approximately half of the complex was dissociated into its constituent M and T subunits existing in charge states similar to those observed in the
case of *Lumbricus* (Fig. 1). It was found that the addition of ammonium acetate to the sample solutions was essential in order to observe the subassembly at adequate sensitivity, with an optimum concentration of 10 mM. Thorough desalting using the ion exchange beads was also necessary, otherwise the peaks were significantly broader, presumably due to the multiple addition of alkali metal ions, mainly Na⁺. Although these latter ligands could not be directly observed in the subassembly, they were distinguishable in association with the monomer and were significantly reduced after desalting but were not completely eliminated.

**Effect of De-clustering Voltage on Mass**—It was also found
that the measured masses of the subassemblies depended to some extent (<1%) on the amount of de-clustering voltage applied to the ions. De-clustering occurs when the ions are accelerated through a region that is intermediate in pressure between their generation at atmospheric pressure and analysis under high vacuum. This acceleration by a potential across the intermediate pressure region has the effect of imparting internal energy to the ions by collisions with the nitrogen gas in this region, causing them to lose weakly bound ligands at low potentials, followed by more strongly bound ligands as the potential is increased. Thus, at low de-clustering potentials, the mass tends to be higher than at high potentials. Fig. 3 shows a series of mass spectra obtained on the time-of-flight instrument for the *Lumbricus* (Fig. 3A) and *Arenicola* (Fig. 3B) subassemblies that illustrate the way in which the mass and appearance of the subassembly peaks change with the de-clustering voltage. Although the *Lumbricus* subassembly mass decreased linearly from ~215,400 Da at 60 V to ~213,300 Da at 200 V, the mass of the *Arenicola* subassembly decreased linearly from ~205,570 at 60 V to ~205,100 at 120 V and then tended toward an asymptote at ~204,600 Da in the range 180–200 V. In addition, above about 140 V, a series of peaks appeared below the major peak that can be attributed to the loss of up to three heme groups. In the case of *Lumbricus*, discrete peaks corresponding to the loss of heme groups were not observed, although above 140 V, there is an obvious broadening of the peak on the low mass side of its maximum. Also, the heme group itself (m/z 616.5) was observed to appear in the spectra from both samples at about 120 V and then increased in intensity at higher voltage. The decrease in mass with increasing de-clustering voltage is most likely due to the loss of complexed water molecules and possibly some cations. The inability to observe the distinct loss of heme groups in the case of *Lumbricus* could be due in part to the heterogeneity of the components composing the subassembly, which is substantially greater than is the case with *Arenicola*.

**DISCUSSION**

*Lumbricus* Hb contains four globin chains (chains a—d) ranging in mass from 15,962 to 19,390 Da, with the monomer subunit (chain d) existing as three isoforms (d1–d3), and chain a occurring as four glycosylated isoforms (a1—a4); the latter form four different disulfide-bonded trimer subunits with chains b and c (8). *Arenicola* Hb contains eight different globin chains (a1, a2, b1, b2, b3, c, d1, and d2) ranging in mass from 15,922 to 17,032 Da, with the b, c, and d chains forming five of the six possible disulfide-bonded trimers (T1–T5) (c + b1 + d1, c + b1 + d2, c + b2 + d1, c + b2 + d2, and c + b3 + d2) (12). Table 1 shows the range of masses observed for the two subassemblies and the selected representative masses together with the calculated masses for dodecamer subassembly compositions M5T, M6T2, and M3T3 and a hexadecamer subassembly.

**Fig. 3.** ESI mass spectra of the subassemblies from (A) *Lumbricus* and (B) *Arenicola* showing the way in which the mass and peak shape were calculated using the transformation software (28) change with de-clustering voltage over the 60–200 V range.
TABLE I

Measured and calculated masses (in Da) of globin subassemblies

| Subassembly | Lumbiricus Hb          | Arenicola Hb          |
|-------------|------------------------|-----------------------|
| Measured mass range | 213,340–215,390       | 204,580–205,570       |
| Representative measured mass | 214,790          | 204,600              |
| Calculated: T + 9M + 12 hemes | 203,943          | 200,779              |
| Calculated: 2T + 6M + 12 hemes | 208,689           | 202,560              |
| Calculated: 3T + 3M + 12 hemes | 213,436           | 204,340              |
| Calculated: 4T + 4M + 12 hemes | 204,578           | 272,454              |
| Calculated mass range | 212,624–214,176     | 203,844–204,495       |
| Calculated for randomly assembled 3T + M + 12 hemes | 213,436 ± 319     | 204,342 ± 80         |

* For a de-clustering voltage range from 200 to 60 V.
* At a de-clustering voltage of 140 V.
* At a de-clustering voltage of 180–200 V.
* Calculated mass using average, intensity weighted masses of the apo-monomer (M) and apo-trimer (T) subunits: 15,983.2 and 52,696.1 Da for *Lumbricus* (8) and 15,966.7 and 49,680.7 Da for *Arenicola* (12) Hbs, respectively.
* Subassembly proposed by Riggs et al. (22–24) for *Lumbricus* Hb.

M₃T₄ using the globin chain and subunit masses determined previously (8, 12). For the *Lumbricus* Hb subassembly, the mass 214,190 Da at a de-clustering voltage of 140 V was taken to be representative, assuming a behavior similar to that of the *Arenicola* Hb subassembly, which displays minimal heme loss at this voltage. For the *Arenicola* Hb subassembly, the asymptotic mass 204,600 Da was taken to be representative. The results shown in Table I demonstrate unequivocally that the M₃T₃ subassembly compositions provide the best agreement between the calculated masses and the experimental masses, even though the latter are somewhat higher, depending on the de-clustering voltage used. Although adduct formation with alkali metal cations cannot be excluded, the mass differences can be ascribed to noncovalent complexation of *Lumbricus* and *Arenicola* subassemblies with approximately 42 and 14 water molecules, respectively. It is now known that water molecules play an important role in the formation of biomolecular complexes (29). Furthermore, recent ESI-MS studies of complexes formed between SH2 domains from tyrosine kinase Src with tyrosyl phosphopeptides (30) and of insulin hexamers (31) have shown that one to three water molecules in the former case and three to six water molecules in the latter were incorporated into the observed protein complexes.

An additional and very important point that needs to be considered in comparing the experimental masses of the subassemblies with the masses calculated from their subunits is the fact that these subassemblies do not have a unique mass. Because each dodecamer subassembly consists of three monomers and three trimers, there are essentially two levels of mass distribution: (a) one for each of the two subunits; and (b) another for their combination into a dodecamer. In the case of the *Lumbricus* Hb subassembly, there are 10 possible ways of forming three monomers from three different chains (d1–d3), and the four different trimers (chains b and c with each of chains a1–a4) provide 20 possible combinations of three trimers; thus, the total number of possible dodecamer subassemblies (i.e. 3M and 3T) is 200. For the *Arenicola* Hb subassembly, there are 4 possible combinations of 3M from two different monomers (chains a1 and a2) and 35 possible combinations of 3T from five different trimers, leading to 140 possible M₃T₃ subassemblies. Because the proportions of the subunit isoforms are known experimentally from previous ESI-MS studies (8, 12), it is possible to calculate a mass distribution based on a straightforward combinatorial model (see "Appendix"). The calculated histograms for the subassembly masses of the two Hbs incorporating only masses with a probability ≥ 0.002 are shown in Fig. 4. The calculated mass range for the *Lumbricus* Hb subassembly (212,624–214,176 Da) is much greater than that for the *Arenicola* Hb subassembly (203,934–204,495 Da) (Table I). The subassembly masses with the highest probability

![Figure 4](https://example.com/figure4.png)
It is appropriate at this point to briefly discuss the disagreement concerning the structure of *Lumbricus* HB between our group and that of A. F. Riggs that has persisted for over a decade. In 1991, Fushitani and Riggs reassembled at a neutral pH the trimer and monomer subunits isolated by dissociation of the HB at pH > 9, observed a 5.8 S species using sedimentation velocity, and suggested it to be an octamer globin complex *M* 3 *T*. In subsequent experiments, the CO forms of the trimer and monomer subunits isolated by dissociation at an alkaline pH were mixed at a neutral pH to obtain a subassembly with a mass of ~280 kDa as determined by multiple angle laser light scattering, in agreement with a mass of 286 kDa calculated for a hexadecamer *Mt* 12, *Tt* 4 (22–24). Based on a weight proportion of linker chains of 0.163, Riggs and his colleagues have proposed a model of *Lumbricus* HB comprising 12 hexadecamers [d] 4 [abc] 4 (192 globin chains) and 24 linkers, with a calculated mass in agreement with the 4.1 ± 0.1 MDa mass for the native HB determined only by multiple angle laser light scattering (22–24). Apart from the fact that the proposed globin to linker stoichiometry corresponds to iron and heme contents that are higher than those generally observed for over 30 HBL HBs by many different investigators (4, 32), the most telling shortcoming of this model is that the proposed hexadecamer subassembly cannot have a 3-fold symmetry and is thus unable to account for the 12 local 3-fold axes observed in the three-dimensional reconstructions based on cryoelectron microscopy images in frozen, hydrated samples of *Lumbricus* HB obtained by the groups of Van Heel and colleagues (16) and Lamy and colleagues (21). Our findings, which are summarized in Table I, do not provide any support for the Riggs model of the globin subassembly.

It should be pointed out that the three-dimensional reconstructions obtained by De Haas *et al.* for the chlorocruorin from *Eudistylia vancouverii* (17) and the HBs from the leech *Macrobabella decora* (18), the vestimentiferan *Riftia pachyptila* (19), and the deep sea polychaete *Alvinella pompejana* (19) show all 200 masses to be different, varying from 212,623.8 to 214,175.7 Da. The dodecamer 21d2d12t4 has the highest probability (0.041) and a mass of 213,512 Da.

The expected mass of a randomly assembled dodecamer can also be easily calculated from Equation 3. Because the random variables *D* and *T* for all *i* = 1, 2, 3 and *j* = 1, 2, 3, 4 follow the binomial distributions B(3; *p*(d1)), B(3; *p*(a1)), and B(3; *p*(c)), the expected total masses of monomers *d*1, *a*1, and *c* are equal to 3 *m*(d1)*p*(d1) and 3 *m*(a1)*p*(a1), respectively. Therefore, the expected mass of a randomly assembled dodecamer is

\[
\text{Em} = 3(m(d1)p(d1) + m(d2)p(d2) + m(d3)p(d3) + m(a1)p(a1) + m(a2)p(a2) + m(a3)p(a3) + m(c1)p(c1) + m(b) + m(c)) = 213,436 \text{ Da}
\]

The S.D. σ of the mass distribution (3) is given by:

\[
\sigma^2 = \sum m^2(\delta, \tau) \cdot p(\delta, \tau) - \text{Em}^2
\]

(4) where summation ranges over all admissible values of vectors *δ* and *τ*, *m*(δ, τ) = *m*(d1)*δ1* + *m*(d2)*δ2* + *m*(d3)*δ3* + *m*(a1)*τ1* + *m*(a2)*τ2* + *m*(a3)*τ3* + *m*(c)*τ4* + 3*m*(b) + 3*m*(c) + *h* is the mass of a randomly assembled dodecamer with *D* = δ and *T* = τ, and *p*(δ, τ) are defined by Equations 1 and 2, respectively. A calculation using MAPLE provided σ = 319 Da.

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