# A Hierarchy of Disulfide-Bonded Subunits: The Quaternary Structure of *Eudistylia* Chlorocruorin†

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ABSTRACT: The quaternary structure of the cysteine-rich, ∼3500-kDa chlorocruorin (Chl) from the marine polychaete *Eudistylia vancouverii* was investigated using maximum entropy deconvolution of the electrospray ionization mass spectra (ESIMS). The native Chl provided two groups of peaks, at ∼25 and ∼33 kDa, and one peak at ∼66 kDa. ESIMS of the reduced and reduced and carbamidomethylated Chl and of its subunits obtained by HPLC provided the complete subunit composition of the Chl. Two groups of nonglobin linker chains were observed: L1a−f (25 000.4, 25 017.9, 25 039.6, 25 057.0, 25 074.4 and 25 096.8 Da) and L2a−d (25 402.7, 25 446.0, 25 461.6 and 25 478.3Da) (±2.5 Da), with relative intensities L1:L2 = 5:2. Six globin chains were found, a1, a2, and b1−4, with reduced masses of 16 051.5, 16 172.4, 16 853.5, 17 088.9, 17 161.2 and 17 103.6 (±1.0 Da) and relative intensities of 8:4:1:4:2:1, respectively. Disulfide-bonded dimers and a tetramer of globin chains were identified: D1 = a1 + b3 at 33 207.1; D2 at 33 374.1, which had a cysteinylated Cys (a2 + b2 + Cys); and D3 = a1 + b4 at 33 149.4 Da (±3.0 Da), with relative intensities D1:D2:D3 = 5:4:1 and T = a1 + a2 + b1 + b2 at 66 154.8 ± 4.0 Da. A 206-kDa dodecamer subunit obtained by dissociation of the Chl in 4 M urea [Qabar, A. N., et al. (1991) *J. Mol. Biol. 222*, 1109−1129], was found to consist only of tetramers T. A model was proposed for the Chl, based on a dimer:tetramer ratio of 2:1: four 206-kDa dodecamers (trimer of tetramers) and 48 dimers tethered to a framework of 30 L1 and 12 L2 linker chains. The 144 globin chains (2480 kDa) and 42 linker chains (1059 kDa) provide a total mass of 3539 kDa, in good agreement with the 3480 ± 225 kDa determined previously by STEM mass mapping. The hierarchy of disulfide-bonded globin subunits observed for *Eudistylia* Chl provides a built-in heterogeneity of hexagonal bilayer structures.

Chlorocruorins (Chls)[1] are giant "green" hemoglobins, found in four marine polychaete families, containing an altered heme with a formyl substituting for the 3-vinyl group (*1*). The Chls have the HBL appearance and size in electron micrographs characteristic of annelid and vestimentiferan extracellular Hbs, a similar sedimentation coefficient of approximately 60S, and the abnormally low iron content of 0.23 wt % (*2*−*6*). Although their oxygen binding affinities are generally lower than those of the annelid Hbs ($P_{50}$ > 50 Torr), the Chls exhibit equally high cooperativities (Hill coefficient $n_{50}$ > 3) (*7*−*10*).

As a result of studies over the last 20 years, the HBL Hbs and Chls are known to have masses of ∼3600 kDa and to consist of two types of chains: heme-containing 16−17-kDa globin chains and nonglobin, linker chains of 25−28 kDa (*11*, *12*). The amino acid sequences of globin chains of HBL Hbs are clearly related to those of vertebrate and invertebrate Hbs and Mbs (*13*, *14*). The HBL Hbs from various annelid groups and the Chls differ among themselves in the nature of their disulfide-bonded globin subunits. The majority of oligochaete and polychaete Hbs have monomers and disulfide-bonded trimers, the leeches and the vestimentiferans have monomers and disulfide-bonded dimers, and the Chls have disulfide-bonded dimers and tetramers (*5*, *11*). This communication presents the results of a detailed ESIMS study of *Eudistylia vancouverii* Chl and its subunits.

## EXPERIMENTAL PROCEDURES

*Materials.* Live *E. vancouverii* were collected at the Oregon Institute of Marine Biology (Charleston, OR), and the Chl was prepared as described previously (*1*, *15*) in 0.1 M Tris·HCl buffer, pH 7.0, and 1 mM EDTA, using a cocktail of protease inhibitors (no. 1697-498, Biochemical Division, Boehringer-Mannheim Corp., Indianapolis, IN). The dodecamer subunit was prepared by gel filtration at neutral pH, after exposure to 4 M urea (*1*). The concentration of the Chl was determined using extinction coefficients of

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[1] Abbreviations: Hb, hemoglobin; Mb, myoglobin; Chl, chlorocruorin; HBL, hexagonal bilayer; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris·HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylene-diaminetetraacetic acid, sodium salt; DTT, dithiothreitol; Cam, carbamidomethyl group; STEM, scanning transmission electron microscopy; HPLC, high-pressure liquid chromatography; FPLC, fast protein liquid chromatography; ESIMS, electrospray ionization mass spectrometry; MaxEnt, maximum entropy deconvolution; SD, standard deviation.

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2.23 mL $mg^{-1}$ $cm^{-1}$ at 280 nm and 0.472 mL $mg^{-1}$ $cm^{-1}$ at 605 nm (*1*).

*HPLC*. Reversed-phase chromatography was performed using Synchropak RP-P, RP1000, and RP4000 $C_{18}$ columns, 4.6 × 250 mm (SynChrom, Inc., Lafayette, IN) and a Waters gradient HPLC system (Waters Chromatography Division, Millipore Corp., Milford, MA). Linear gradients of water−acetonitrile in 0.1% aqueous trifluoroacetic acid were used with flow rates between 0.4 and 0.8 mL/min. The absorbance of the eluate was monitored at either 220 or 400 nm. The chromatographic solvents were HPLC reagent grade (J. T. Baker, Inc., Phillipsburg, NJ).

*Electrospray Ionization Mass Spectrometry*. The data were acquired on a Quattro II electrospray mass spectrometer (Micromass U.K., Ltd., Altrincham, Cheshire, U.K.), using sample concentrations of 0.25−0.5 $\mu$g/$\mu$L in 50% aqueous acetonitrile containing 0.2% formic acid. The sample flow rate into the electrospray source was 5 $\mu$L/min, and data were typically acquired over 5−10 min. ESIMS 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, $m/z = (M + nH^+)/n$, where M 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 the Hb data contain several proteins, each producing 5−10 multiply charged ions in a series, they were processed 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, U.K.) incorporated as part of the Micromass MassLynx software suite on a 60-MHz Pentium PC supplied with the spectrometer. Mass scale calibration employed the multiply charged series from horse heart myoglobin (Sigma Chemical Co., St. Louis, MO), using a calculated mass of 16 951.5 Da (*18*), based on the following atomic weights: C = 12.011, H = 1.00794, N = 14.00674, O = 15.9994, and S = 32.066 (*19*).

*Preparation of Samples for ESIMS*. A stock solution of Hb was made in water or 0.05−0.3% aqueous formic acid to give a concentration of 5 $\mu$g/$\mu$L. Working solutions of native Hb, actually denatured native Hb, were 0.25−0.5 $\mu$g/$\mu$L in 50% aqueous acetonitrile containing 0.2% formic acid. Carbamidomethylated Hb was prepared by mixing 10 $\mu$L of Hb stock solution, 2 $\mu$L of 1 M aqueous ammonium bicarbonate, 2.5 $\mu$L of 0.2 M aqueous iodoacetamide solution, and 20 $\mu$L water, allowing the mixture to stand for 10 min, and then adding 20 $\mu$L of 1.0% formic acid and 50 $\mu$L of acetonitrile to make the solution for ESIMS analysis. Reduction was effected by adding 10 $\mu$L of 0.1 M DTT to a 50-$\mu$L aliquot of the Hb stock solution mixed with 30 $\mu$L of water and 10 $\mu$L of 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-$\mu$L aliquots were removed. To one, 20 $\mu$L of 1.0% formic acid, 20 $\mu$L of water, and 50 $\mu$L of acetonitrile were added to provide a working solution of reduced Hb. To the other aliquot, 2.5 $\mu$L of 0.2 M iodoacetamide and 20 $\mu$L water were added, mixed and allowed to stand at room temperature for 10min. Then 20 $\mu$L of 1% formic acid and 50 $\mu$L acetonitrile were added to make a working solution for ESIMS analysis of reduced, carbamidomethylated Hb.

## RESULTS

*ESIMS of Native and Reduced Chl*. The native Hb was first analyzed in denaturing solvent to determine the masses of the components that exist as covalently bound entities, i.e., as linker chains and disulfide-bonded multimeric subunits. It was then reduced with DTT to determine the masses of the globin chains that compose the multimeric subunits. The ESIMS multiply charged spectra of native Chl, when processed using the MaxEnt program, produced zero-charge spectra on a true mass scale, which showed the presence of two groups of peaks, one at 25−26 kDa consisting of nonglobin linker chains and another at 33−34 kDa, with an additional peak at ∼66 kDa in some cases (Figure 1A). The two groups of peaks are shown in spectra B and C of Figure 1 on expanded mass scales. Table 1 provides the masses and relative intensities of the 10 observed linker peaks in the 25−26-kDa range. The relative intensities of the two groups of linkers are L1:L2 = 3.19:1.30. Reduction with DTT for 5 min led to the disappearance of almost all the peaks at ∼25 kDa; the three peaks D1−D3 at 33 207.7, 33 373.8 and 33 150.8 Da; and the peak at 66 154.8 Da with the concomitant appearance of a new group of peaks at 16−17 kDa and of a single peak D2′ at 33 258.8 Da (Figure 2A), indicating that the peaks at ∼33 and 66 kDa are disulfide-bonded dimers and tetramers of globin chains, respectively. Figure 2B shows the 16−17-kDa region after a 10-min reduction, using an expanded mass scale. The masses and relative intensities of the six globin peaks a1, a2, and b1−b4 are provided in Table 2. None of the observed peaks had satellite peaks differing by ∼162 Da, indicating the absence of glycosylation, in agreement with earlier findings (*1*).

*ESIMS of Native/Cam and Reduced/Cam Chl*. The native Hb was carbamidomethylated and analyzed by ESIMS to determine the number of free Cys residues associated with each subunit. It was then reduced and carbamidomethylated to determine the total number of Cys residues present in each globin and linker chain. Carbamidomethylation of the Chl resulted in altered locations of the three linker peaks L2b−L2d to 25 501.6, 25 518.7, and 25 538.4 Da, respectively (Table 1) and of the disulfide-bonded dimer D1 to 33 264.3Da (Table 3). The mass differences, mean 57.5 ± 1.9 Da, correspond to the addition of one Cam group to L2b−L2d and D1, implying the presence of one free Cys in each subunit. Reduction of the Chl followed by carbamidomethylation resulted in extensive modification of the masses of the globin chains a1, a2, and b1−b4 as shown in Table 2. The number of Cys residues present in each globin chain was calculated from the difference between the reduced and the reduced/carbamidomethylated masses.

*ESIMS of Chl Subunits*. The dodecamer subunit isolated by gel filtration of the Chl dissociated in 4 M urea (*1*), when subjected to ESIMS in the unreduced and reduced forms, was found to contain chains a1, a2, b1, and b2 (Figure 2C).

Figure 3 shows an HPLC chromatogram of the Chl. Peak H represents the heme that is dissociated at the acid pH. SDS−PAGE showed that peaks 1−8 corresponded to the linkers, peaks G1 and G3 corresponded to reducible dimers, and peaks G2 and G4 corresponded to the tetramer band, observed earlier in the unreduced SDS−PAGE patterns of the Chl (*1*).

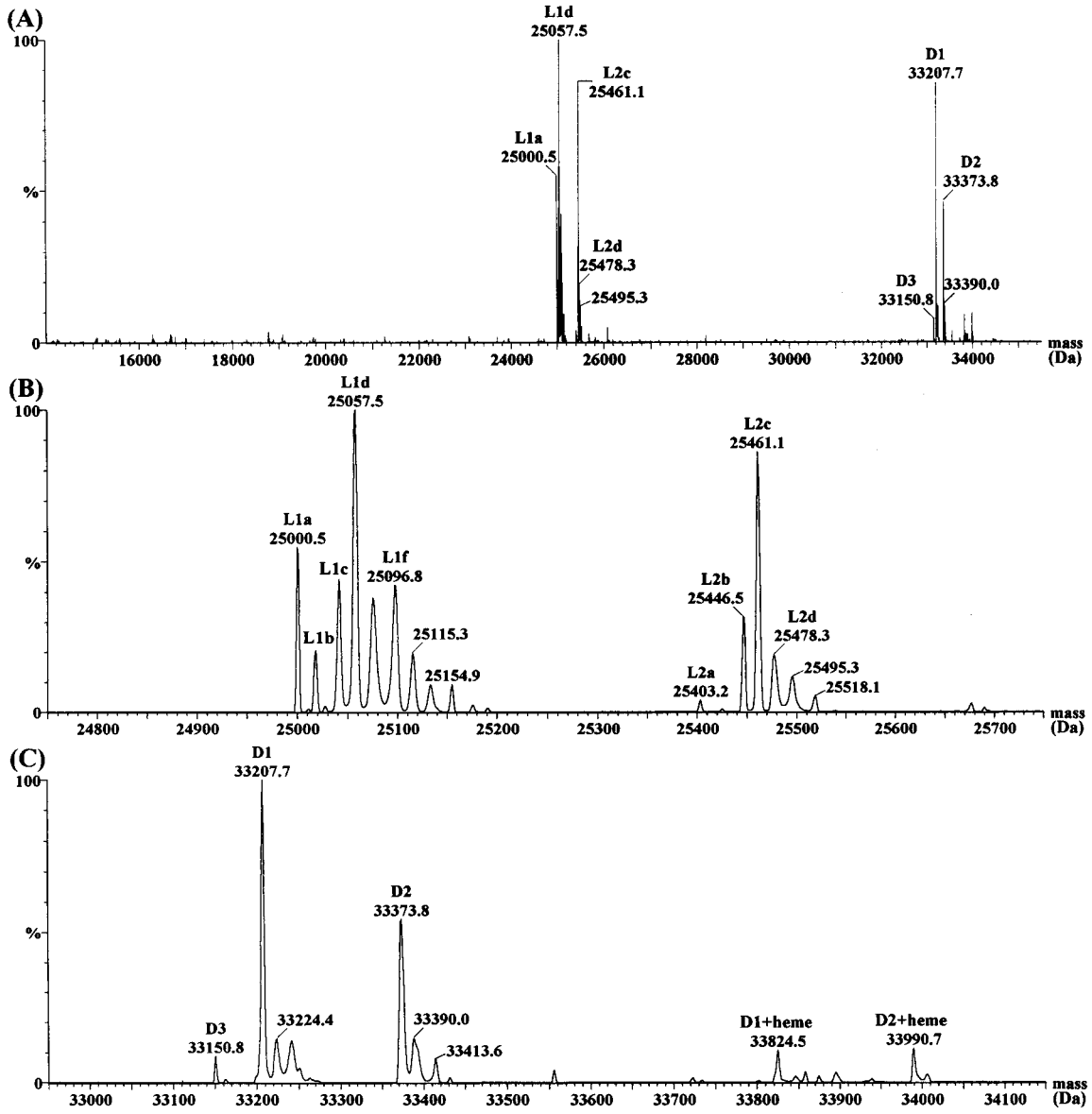


FIGURE 1: MaxEnt-processed ESIMS spectra of native *Eudistylia* Chl: (A) the overall spectrum, (B) the 25−26-kDa region, and (C) the 33−34-kDa region using expanded mass scales.

Table 1: Summary of ESIMS Masses of *Eudistylia* Chl Linker Chains[a]

| | | measured mass | | |
|---|---|---|---|---|
| chain | rel intensity | native Hb[b] | Cam[c] | free Cys |
| L1a | 0.34 | 25 000.4 | 25 000.1 | 0 |
| L1b | 0.17 | 25 017.9 | 25 017.2 | 0 |
| L1c | 0.44 | 25 039.6 | 25 040.0 | 0 |
| L1d | 1.00 | 25 057.0 | 25 057.2 | 0 |
| L1e | 0.63 | 25 074.4 | 25 074.3 | 0 |
| L1f | 0.61 | 25 096.8 | 25 095.2 | 0 |
| L2a | 0.06 | 25 402.7 | N.O. | |
| L2b | 0.25 | 25 446.0 | 25 501.6 | 1 |
| L2c | 0.73 | 25 461.6 | 25 518.7 | 1 |
| L2d | 0.26 | 25 478.3 | 25 538.4 | 1 |

[a] In daltons; estimated error is ±2.5 Da. N.O., not observed. [b] Mean of 5 determinations on native Chl. [c] Mean of 2 determinations on carbamidomethylated Chl.

The linker peaks 1−8 (Figure 3) provided inconclusive results when subjected to ESIMS: peak 3 contained the L2 group of linker chains, and peaks 4 and 5 consisted predominantly of linker chains L1. Reduction and carbamidomethylation of HPLC peaks 5 and 3 demonstrated that the major components L1d and L2c contain at least 10 and 9 Cys residues, respectively.

ESIMS of the globin peaks G1−4 (Figure 3) showed that G1 consisted of the disulfide-bonded dimer D2, G2 comprised only the tetramer at 66 155.5 Da, and both G3 and G4 consisted mainly (>90%) of dimer D1. In G4, a tetrameric component, attributable from its mass to dimeric D1, was also detected at about 10% of D1. ESIMS, upon reduction, confirmed the composition of G1 (D2, chains a2 and b2) and G3 and G4 (D1, chains a1 and b3) and revealed that G2, i.e., the tetramer, consisted of chains a1, a2, b1, and b2. None of the globin peaks obtained by HPLC included the dimer D3 observed in the ESIMS spectra of native and reduced Chl.

Figure 4 shows the spectra obtained with peak G1 (dimer D2) as a function of reduction time with 10 mM DTT. With progressive reduction, the appearance of chains a2 and b2 was accompanied by the formation of peak D2′ at 33 259

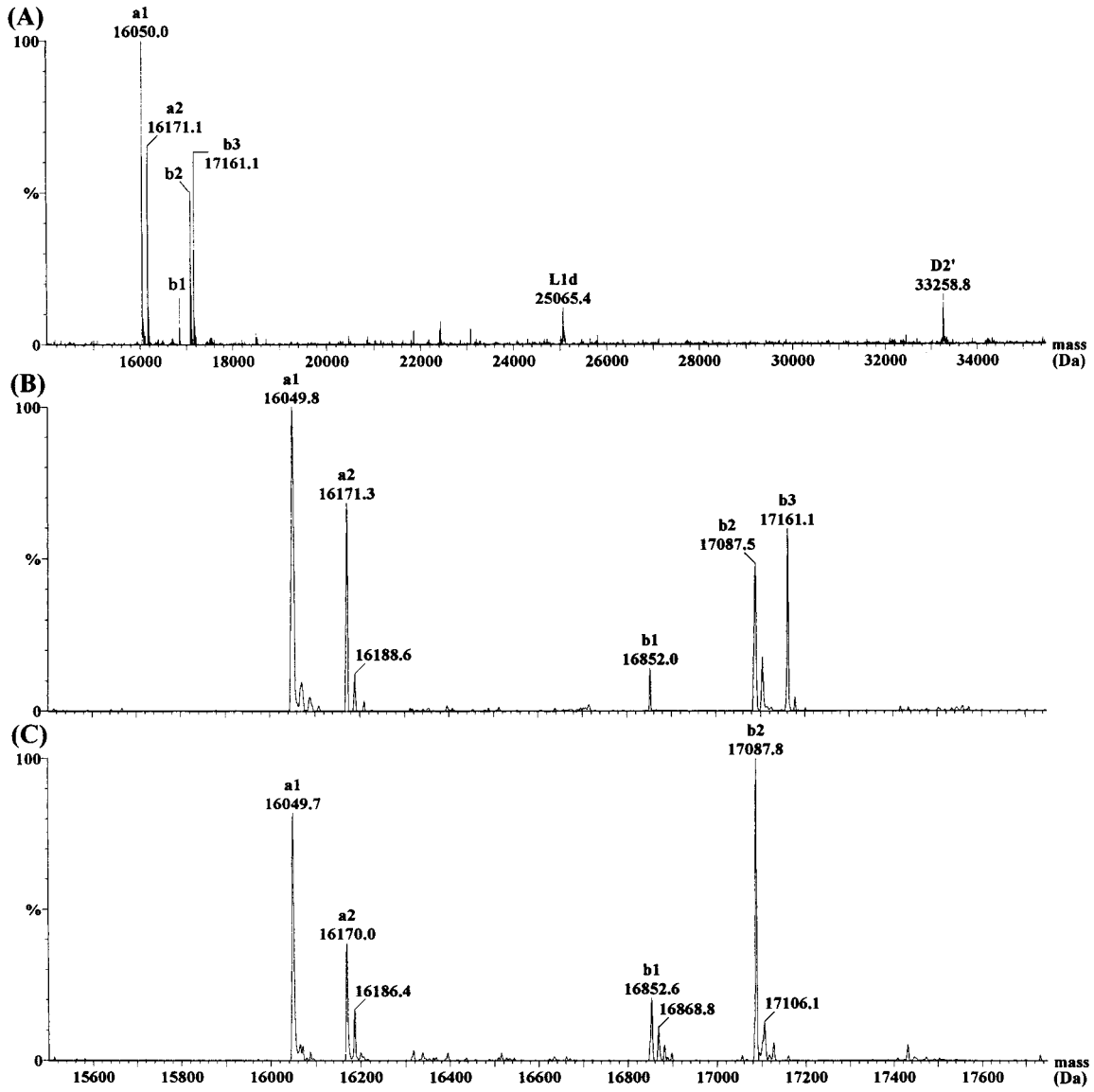


FIGURE 2: MaxEn-processed ESIMS spectra: (A) the overall spectrum of *Eudistylia* Chl in the reduced state (10 mM DTT, 5 min), (B) the 16−17-kDa region of the reduced Chl (10 mM DTT, 10 min) using an expanded mass scale, and (C) the 16−17-kDa region of the reduced dodecamer subunit (10 mM DTT, 10 min) using an expanded mass scale.

Table 2: Summary of ESIMS Masses of *Eudistylia* Chl Globin Chains[a]

| chain | rel intensity[b] | measured mass red Hb[c] | measured mass red/Cam[d] | cor mass[e] | no. of Cys[f] |
|---|---|---|---|---|---|
| a1 | 1.00 | 16 049.6 ± 0.2 | 16 222.7 ± 0.1 | 16 051.5 ± 0.1 | 3 |
| a2 | 0.40 | 16 171.0 ± 0.3 | 16 343.6 ± 0.4 | 16 172.4 ± 0.4 | 3 |
| b1 | 0.06 | 16 852.6 ± 0.4 | 17 138.8 ± 0.3 | 16 853.5 ± 0.3 | 5 |
| b2 | 0.39 | 17 087.3 ± 0.5 | 17 317.1 ± 0.6 | 17 088.9 ± 0.6 | 4 |
| b3 | 0.32 | 17 161.1 ± 0.2 | 17 389.4 ± 0.7 | 17 161.2 ± 0.7 | 4 |
| b4 | 0.12 | 17 103.6 ± 0.8 | N.D. | N.D. | N.D. |

[a] Masses in daltons; estimated error is ±1.5 Da. N.D., not determined. [b] Relative intensities from reduced native Chl. [c] Mean of 7 determinations ±SD on reduced native Chl. [d] Mean of 4 determinations ±SD on reduced/carbamidomethylated Chl. [e] Corrected for carbamidomethylation (red/Cam masses, 57.052 Da/Cys). Masses are calculated with Cys reduced. [f] Total number of Cys = (red/Cam mass − red mass)/57.052.

Da, assigned to decysteinylated D2, D2-Cys (Table 4), with the concomitant disappearance of the D2 peak. Similar experiments with peak G2 (subunit T) showed that the tetramer peak disappeared after a 1-min reduction with the concomitant appearance of chains a1, a2, b1, and b2 and a peak with the same mass as D2′ produced by reducing the dimer D2. On further reduction, the intensity of D2′ decreased and the intensities of the globin chains increased. This implies that D2′ is produced, albeit as a transitory entity, by reducing both D2 and T and provides evidence for chains a2 and b2 being disulfide-bonded together in the tetramer. Carbamidomethylation of G2 established the presence of one free Cys in the tetramer subunit. Tables 3 and 4 summarize the ESIMS masses and compositions of the dimer and tetramer subunits based on the data in Table 2.

*Relative Stoichiometry of the Globin Subunits*. We used the HPLC data illustrated in Figure 3 to estimate the relative stoichiometry of the dimer and tetramer globin subunits from the sums of the relevant peak areas obtained by integration and by assuming that the extinction coefficients were equal. Peaks G1 and G2 were identified to consist of dimers D2 and tetramers T, respectively (see above). Since peaks G3 and G4 consist only of dimers D1, it is likely that they represent the monomeric D1 and the dimeric D1 (tetramers

Table 3: Summary of ESI-ms Masses of *Eudistylia* Chl Globin Subunits[a]

| subunit | rel intensity | native Hb[b] | measured mass | | HPLC/Cam[e] | cor mass[f] | free Cys |
|---|---|---|---|---|---|---|---|
| | | | Cam[c] | HPLC[d] | | | |
| dimer D1 | 1.0 | 33 207.2 ± 0.3 | 33 264.3 ± 0.3 | 33 207.0 ± 0.4 | N.D. | 33 207.2 | 1 |
| dimer D2 | 0.8 | 33 373.8 ± 0.6 | 33 372.3 ± 0.4 | 33 374.8 ± 2.1 | N.D. | | 0 |
| dimer D3 | 0.1 | 33 149.7 ± 1.0 | N.D. | 33 148.9 ± 0.3 | N.D. | | N.D. |
| tetramer T | N.D. | 66 154.8 | N.D. | 66 155.5 ± 2.8 | 66 210.5 ± 2.5 | 66 153.5 ± 2.5 | 1 |

[a] Masses in daltons; estimated error is ±3.0 Da for dimers and ±4.0 Da for tetramers. N.D., not determined. [b] Mean of 5 determinations ±SD on native Hb except for T (one value only). [c] Mean of 2 determinations ±SD on carbamidomethylated native Chl. [d] Mean of 3, 2, and 7 determinations ±SD on HPLC-isolated D1/D3, D2, and T, respectively. [e] Mean of 4 determination ±SD on HPLC-isolated and carbamidomethylated tetramer. [f] Corrected for carbamidomethylation (Cam mass, 57.052 Da/Cys).

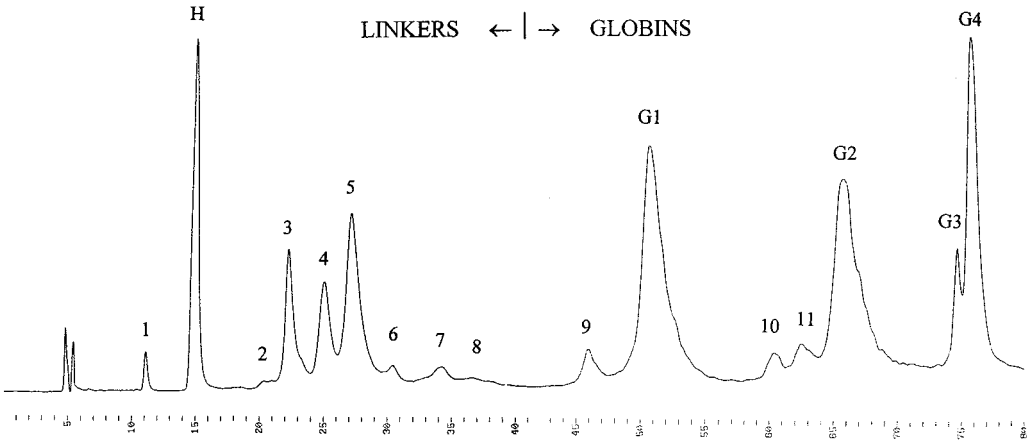


FIGURE 3: Reversed-phase chromatogram of the Chl obtained with a 4.6 × 250 mm $C_{18}$ Synchropak RP-P column and two separate, consecutive gradients of acetonitrile in aqueous 0.1% TFA (first 40 min, 35−45% acetonitrile; next 40 min, 45−52% acetonitrile). Ordinate, absorbance at 220 nm in arbitrary units; abcissa, time in minutes. Note that the division of the chromatogram into linker and globin regions is purely operational; all the linkers emerged before any of the globin subunits. SDS−PAGE of the combined peaks 1−8 and of the combined peaks 9−11 and G1−G4 corresponded to the known pattern obtained earlier by Qabar et al. (*1*).

$[D1]_2$), respectively. If we assume that the minor peak 9 also consists of D2 and that the minor peaks 10 and 11 are comprised of tetramers T, we obtain the ratios D1:T:D2 [$\sum$(peak 9 + G1):$\sum$(peaks 10 + 11 + G2):$\sum$(peaksG3 + G4)] = 0.36:0.37:0.28 (SD ± 0.02, $N = 35$). The ratio D1:D2 = 1.0:1.3 (0.28:0.36) is in disagreement with the ratio 1.0:0.8 indicated by the ESIMS intensities (Table 3); a ratio closer to 1:1 is obtained if the areas of the subsidiary peaks 9−11 are not included. Overall, the ratio of disulfide-bonded dimer subunits to disulfide-bonded tetramer subunits is [D1 + D2]:T = 0.64:0.37 = 1.7:1 (or 0.69:0.32 = 2.2:1, if peaks 9−11 are not included). These results suggest that the relative stoichiometry of the dimers and tetramers, D1:D2:T, is approximately 1:1:1.

*Linker-to-Globin Ratio.* The HPLC data were also used to determine the ratio of the linker subunits to the globin subunits from the sums of their respective peak areas, $\sum$(peaks 1−8):$\sum$(peaks 9−11) + $\sum$(peaks G1−G4)] (Figure 3). The calculated mean ratio was 0.275:0.725 (SD ± 0.03, $N = 35$). If the outliers at ±1 SD of the mean are left out, the new mean is 0.27:0.73 (SD ±0.01, $N = 26$). Since we were able to identify only peaks 3, 4, and 5 and peaks G1−G4 by ESIMS, we also calculated the ratio $\sum$(peaks 3−5):$\sum$(peaks G1−G4); the result, 0.27:0.73 (SD ±0.02) suggests that the contribution of the unidentified minor peaks is not significant. The HPLC results are in agreement with earlier results, ranging from 0.7:0.3 to 0.75:0.25, obtained previously (*1*) and with the corresponding properties of other HBL Hbs and Chls (*6*, *12*).

## DISCUSSION

In examining the ESIMS results (Figures 1 and 2), it is obvious that several of the observed major peaks are accompanied by higher mass satellites of much lower intensity. These are generally adducts with metal ions, such as $Na^+$ and $K^+$, as well as adducts with $\Delta M = +16-18$ Da due to oxidation or hydroxylation (*20*). Loss of water as well as facile gas-phase cleavage at Asp−Pro, Asp−Met, and other Asp−Xxx peptide bonds are also known to occur (*21*−*23*). Generally, the low-intensity satellite peaks are ignored, since their appearance and intensity vary from one experiment to another.

The very high resolution afforded by the MaxEnt deconvolution of the raw ESIMS spectra (*24*) has permitted an unambiguous and self-consistent determination of the number of different chains and subunits comprising *Eudistylia* Chl. Tables 1 and 2 summarize the masses and relative intensities of the linker and globin chains, respectively. The six globin and 10 linker chains identified in this case represent substantially more variability than has been observed heretofore in the ESIMS studies of HBL Hbs from *Tylorrhynchus* (*25*), *Macrobdella* (*26*), *Lumbricus* (*27*), *Riftia* (*28*), and *Arenicola* (*29*). We do not know whether other Chls are as complicated as that of *Eudistylia*.

A puzzling observation was the disappearance of or the severe reduction in the relative intensities of the linker chains from the ESIMS spectra of reduced Chl (Figure 2A), a phenomenon that has not been observed previously with HBL Hbs and for which we have no explanation. However, the

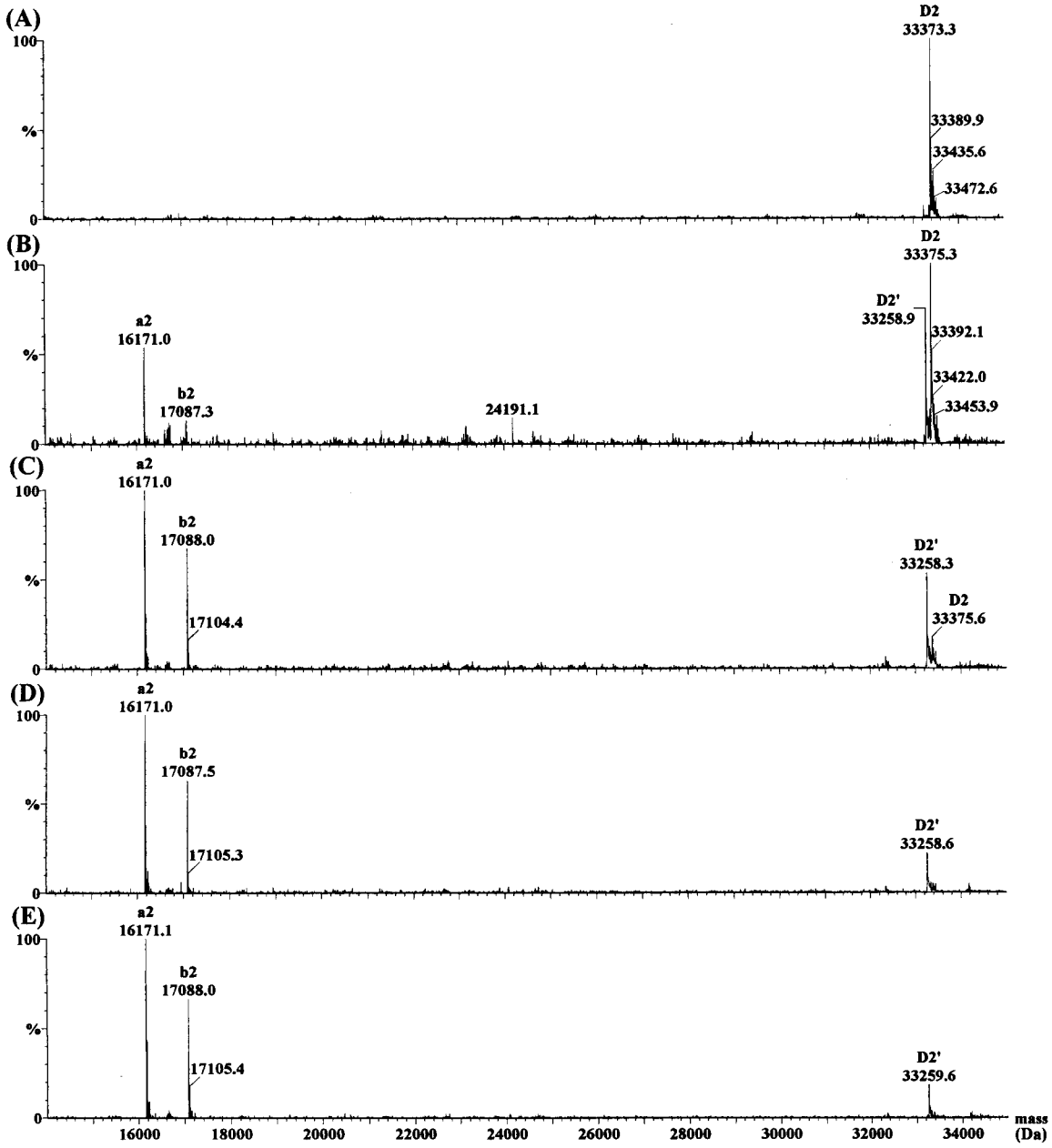


FIGURE 4: MaxEnt-processed ESIMS spectra of peak G1 obtained by HPLC of native *Eudistylia* Chl: (A) unreduced and (B−E) reduced for 1, 5, 10, and 20 min, respectively, with 10 mM DTT.

Table 4: Chain Assignments of *Eudistylia* Chl Globin Subunits[a]

| | | | | | disulfide bonds | | |
|---|---|---|---|---|---|---|---|
| subunit | assignment | calcd mass[b] | measured mass[c] | nass D[d] | intra | inter | free Cys |
| D1 | a1 + b3−6H | 33 206.7 | 33 207.1 | 0.4 | 2 | 1 | 1 |
| D2 | a2 + b2−6H + Cys | 33 374.4 | 33 374.1 | −0.3 | 2 | 1 | 0 |
| D2′ | a2 + b2−6H | 33 255.3 | 33 257.4[e] | 2.1 | 2 | 1 | 1 |
| D3 | a1 + b4−6H | 33 149.1 | 33 149.4 | 0.3 | 2 | 1 | ND |
| T | a1 + a2 + b1 + b2 − 14H | 66 152.2 | 66 154.8 | 2.6 | 4 | 3 | 1 |

[a] Masses in daltons. [b] Using corrected masses (Table 2), except for b4, which uses the mass from reduced Chl. [c] Mean of all available measurements on native Chl and HPLC fractions. [d] Difference between calculated and measured mass. [e] From two measurements each on native Chl and HPLC-isolated D2 after 1 min in 10 mM DTT.

results of reducing and carbamidomethylating the two groups of linker chains isolated by HPLC showed that the major components of L1 and L2 have at least 10 and 9 Cys residues, respectively. This is in accord with previous findings from several HBL Hbs which indicate that the linker chains generally contain 9−12 Cys residues (*12*).

Furthermore, we were able to determine the number of Cys residues in globin chains a1, a2, and b1−b3, as well as the number of Cys residues engaged in inter- and intra-disulfide bonds in the dimer subunits D1−D3 and the tetramer T. Single free Cys residues were found in dimer D1 and tetramer T but not in dimer D2. Our assignments

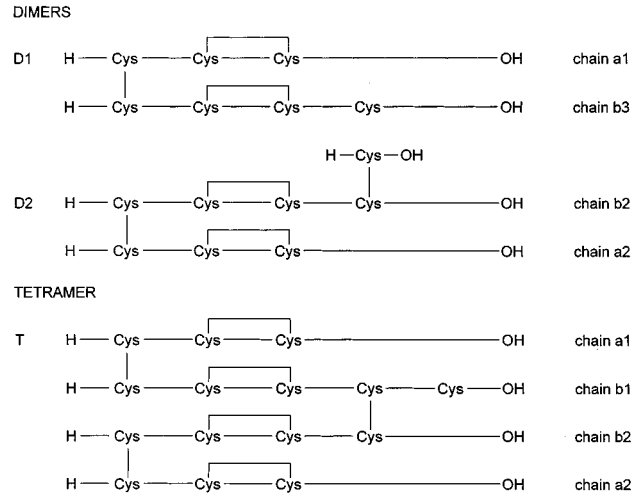


FIGURE 5: Diagrammatic representations of the dimer and tetramer subunits based on the ESIMS results.

of the globin chain compositions of the observed dimer and tetramer subunits are presented in Table 4. The self-consistency of the assignments is quite good and well within the estimated errors for the ESIMS determinations, except, apparently, for D2′. In this case, the mass difference between D2 in native Chl and D2′ in reduced Chl is 116.7 Da (Table 4), whereas the calculated mass difference for a Cys bound to one of the Cys residues in D2 through an interchain disulfide bond (cysteinylation) is 119.1 Da. This discrepancy can be explained by a partial reduction of intrachain disulfide bonds in D2′, since D2′ was only observed after reduction. The assignment of D2 (and hence D2′) given in Table 4 is strongly supported by the excellent agreement (−0.3 Da) between the measured mass of D2 and its mass calculated from the reduced masses of its components plus cysteinylation. The evolution of the ESIMS spectra of D2, isolated as HPLC peak G1, as a function of reduction time (Figure 4) clearly indicates that the cysteinylated Cys in D2 is more easily reduced than the interchain bond holding chains a2 and b2 together. Obviously the fourth Cys residue in chain b2 is the one that is cysteinylated. Although dimer D3, a minor component, also has 7 Cys residues and two intra-disulfide bonds and one inter-disulfide bond such as D1 and D2, we were unable to determine the state of its seventh Cys.

Only one tetramer species was observed in the ESIMS of native Chl, corresponding to subunit G2 isolated by HPLC. Although its most plausible composition is a1 + a2 + b1 + b2 (Table 4), there are three other combinations of chains a1, a2, b1, and b2 which could fit the measured mass of the tetramer T: 2a1 + 2b1 − 12H + 3Cys = 66 155.3 Da; 2a2 + 2b1 − 14H + Cys = 66 156.8 Da; and a1 + a2 + 2b1 − 14H + 2Cys = 66 155.1 Da. Since the last combination lacks a free Cys and, together with the other two, does not contain all four chains, we believe our assignment to be correct. It should be noted that HPLC and SDS−PAGE data indicate that tetramers are also formed by the dimer D1; such a tetramer would have a mass of 66 411.4 Da. Since it was not observed in the ESIMS of native Chl and since it appears to represent only about 10% of dimer D1 in peak G4 (Figure 3), it is likely to be the result of some reshuffling of disulfide bonds in D1. Figure 5 shows schematic diagrams of the disulfide bonding in dimers D1 and D2 and tetramer T that are consistent with the ESIMS results.

Our earlier detailed study of the dissociation of *Eudistylia* Chl has shown that, in addition to the linker subunits, dodecamers (∼200 kDa), tetramers (∼66 kDa), and dimers (∼33 kDa) of globin chains were produced by partial as well as total dissociation in the presence of urea and SDS (*1*). We propose that the largest observed subunit, the dodecamer, is a noncovalently bonded trimer of the disulfide-bonded tetramer T (3[a1 + a2 + b1 + b2]) with 12 heme groups and a mass of 205 863 Da [(3 × 66 155) + (12 × 616.5)]. Although we had speculated previously that the disulfide-bonded dimer subunits could dimerize to tetramers which in turn could also form dodecamers, the ESIMS results obtained here have ruled out this possibility. The tetramer subunits observed previously are likely to be noncovalent hetero- and possibly homodimers of subunits D1−D3 and 4 heme groups ([D1 + D2], 69 047 Da; [D1 + D3], 66 822 Da; [D2 + D3], 68 990 Da; $[D1]_2$, 68 880 Da; $[D2]_2$, 69 215 Da; $[D3]_2$, 68 764 Da). From our present results, we have evidence only for the noncovalent homodimerization of D1 (peaks G3 and G4 in Figure 3).

Recent cryoelectron microscopic studies of *Lumbricus* Hb (*30, 31*), *Eudistylia* Chl (*32*), *Macrobdella* Hb (*33*), *Riftia* Hb (*34*), and *Alvinella* Hb (*35*) have demonstrated that at the level of the resolution achieved, 25−35 Å, all the reconstruction volumes were remarkably alike, implying that

Table 5: Proposed Model of the Quaternary Structure of *Eudistylia* Chl

| subunit | heme groups/subunit | mass (kDa) | no. of copies | no. of heme groups | mass contribution (kDa) |
|---|---|---|---|---|---|
| dodecamers[a] | 12 | 205.9[b] | 4 | 48 | 824 |
| dimers | 2 | 34.5[c] | 48 | 96 | 1656 |
| linkers | | | | | |
| L1 | 0 | 25.1[d] | 30 | | 753 |
| L2 | 0 | 25.5[d] | 12 | | 306 |
| | | total globin mass | | 2480 | |
| | | total linker mass | | 1059 | |
| | | total Chl mass | | 3539 | |
| | | exptl Chl mass[e] | | 3480 ± 225 | |
| | | calcd globin:linker ratio | | 0.701:0.299 | |
| | | exptl globin:linker ratio | | 0.725:0.275 ± 0.029 | |

[a] Each dodecamer consists of three tetramers, T. [b] Calculated for $T_3$ from Table 3, including heme groups (616.5 Da): 3 × 66.155 + 12 × 0.6165. [c] From Table 3, including heme groups. [d] From Table 1. [e] Determined by STEM mass mapping (*1*).

their quaternary structures must also be very similar.

ESIMS investigations of the Hbs from *Tylorrhynchus*, *Macrobdella*, *Lumbricus*, *Riftia*. and *Arenicola* (*25*−*29*) have provided accurate masses of the constituent linker and globin subunits. In all cases, the bracelet model consisting of 12 dodecamers (144 globin chains) tethered to a central hexagonal complex of 36 or 42 linker chains, proposed earlier for *Lumbricus* Hb (*36*, *37*), fits very well the known masses of the native Hbs and is strongly supported by the three-dimensional reconstructions obtained by cryoelectron microscopy.

In Table 5, we present a model of the Chl quaternary structure based on the ESIMS and HPLC results: weighted masses of 25.1 kDa for L1 and 25.5 kDa for L2, a ratio of L1:L2 = 2.5:1 (3.19:1.30 from ESIMS relative intensities in Table 1), a weighted mass of 34.5 kDa for the dimer subunits, a mass of 206 kDa for the dodecamer subunit based on the tetramer mass, a 2:1 stoichiometry of dimer to tetramer, and a linker:(dimer + tetramer) ratio of 0.275:0.725. Furthermore, we assume that the number of copies of L1 and L2 should be multiples of 6 in view of the evident 6-fold symmetry of the HBL structure. Our model comprises 144 globin chains distributed among 4 dodecamers (12 tetramers) and 48 dimers, 30 L1 linkers, and 12 L2 linkers. The total calculated mass is 3539 kDa, in excellent agreement with the mass of the native Chl determined by STEM mass mapping, 3480 ± 225 kDa (*1*). Although the proposed model for *Eudistylia* Chl is very similar to the models put forward by us for *Lumbricus* and several other HBL Hbs based on detailed ESIMS polypeptide chain and subunit compositions, extensive STEM mass mapping, and sedimentation equilibrium studies (*12*), it is unique in the implied heterogeneity of the Chl quaternary structure. We do not know whether the disulfide-bonded dimers form noncovalent dodecamer complexes that are quasi-equivalent to the dodecamers formed from tetramers, nor do we know whether the number of L1 and L2 linkers is constant for all Chl HBL structures. Assuming that it is, native Chl may be a mixture of HBL structures ranging from those consisting of 12 dodecamers to those consisting of 72 dimers. In summary, *Eudistylia* Chl comprises a novel hierarchy of disulfide-bonded globin subunits ranging from the dodecamer to the dimer, with a built-in heterogeneity of possible HBL quaternary structures.

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