Investigation by Electrospray Ionization Mass Spectrometry of the Extracellular Hemoglobin from the Polychaete Annelid *Alvinella pompejana*: An Unusual Hexagonal Bilayer Hemoglobin†

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ABSTRACT: *Alvinella pompejana* inhabits deep-sea hydrothermal vent sites along the East-Pacific Rise, where it colonizes the walls of actively venting high-temperature chimneys. This worm is the most thermophilic metazoan known to date. In *Alvinella*, as in other alvinellids, oxygen transport is mainly achieved by an extracellular Hb dissolved in the vascular blood. This Hb has a molecular mass of 3833 ± 14 kDa as revealed by multiangle laser light scattering (MALLS). Native and derivative Hb (reduced, carbamidomethylated, and deglycosylated) were analyzed by electrospray ionization mass spectrometry (ESI-MS). The data were processed by the maximum entropy deconvolution system (MaxEnt). We identified three groups of peaks for *Alvinella* Hb, at *ca.* 16, 23−26, and 50 kDa corresponding to (i) four monomeric globin chains, *a1* (16 633.4), *a2* (16 532.4), *a3* (16 419.6), and *a4* (16 348.9); (ii) four linker chains, *L1*−*L4* (22 887.1, 24 230.5, 26 233.6, and 25 974.4); and (iii) one disulfide-bonded trimer *T* (51 431.9) composed of globin chains *b* (16 477.5), *c* (16 916.1), and *d* (18 048.8). These Hbs were also subjected to SDS−PAGE analysis for comparative purposes. In addition, using the ESI-MS data we propose two alternative models for the quaternary structure of *Alvinella*'s Hb.

Extracellular hemoglobins (Hb)[1] of a quite specific shape are present in all three annelid classes and vestimentiferan phylum [reviewed in Terwilliger (1992) and Lamy et al. (1996)]. These proteins show a characteristic two-tiered hexagonal structure when negatively stained and observed under transmission electron microscope. They possess a high molecular mass, ranging from 3000 to 4000 kDa (*i.e.*, 60S), as well as an acidic isoelectric point and low heme and iron contents (Vinogradov et al., 1982; Vinogradov, 1985a,b). To comprehend the quaternary structure and to conceive models of these giant Hbs has been a challenge over several years, aiming to understand the structure−function relationships of these hetero-multimeric complexes (Chung & Ellerton, 1979; Garlick & Riggs, 1982; Suzuki & Gotoh, 1986; Suzuki et al., 1989; Vinogradov et al., 1986, 1991; Ownby et al., 1993). Even though a total agreement has not yet been attained, a general consensus emerges from these studies. All HBL Hbs examined so far are built from 12 subunits composed of globin chains (*ca.* 17 kDa) and linked together by a complex central structure called linker chains (*ca.* 24−32 kDa) (Vinogradov, 1985b, Vinogradov et al., 1986, 1991; Martin et al., 1996; Zal et al., 1996a, 1997a).

The Pompeii worm *Alvinella pompejana* Desbruyères & Laubier, 1980 is a tubicolous polychaete annelid which lives in whitish honeycomb-like structures built in the hottest part of the hydrothermal vent ecosystem. At this level, the temperature gradient between the deep sea water (2 °C) and the hot (350 °C) hydrothermal fluid is extremely variable. Although it is unclear where the worms exactly reside in the settlement structure, some observations have reported individuals living around 50 °C (Baross & Deming, 1985) and recently Chevaldonné et al. (1992) report the observation of an *Alvinella* specimen coiled around the tip of the American submersible's high-temperature probe, which simultaneously recorded 105 °C. These authors concluded that *A. pompejana* was probably the most thermophilic metazoan known to date.

The alvinellids possess well developed gills (Jouin & Gaill, 1990) and a closed vascular system containing an extracellular Hb dissolved in the blood. Recently, Jouin-Toulmond et al. (1996) have studied the circulatory system of several alvinellids and revealed the presence of an intracellular Hb contained in their coelomic fluid. The structure of the extracellular Hb has barely been studied in detail due to the difficulty to collect this species. Terwilliger and Terwilliger (1984) showed that *Alvinella* Hb possessed a structure typical of non-vent annelid HBL Hbs. They also reported the very high stability of *Alvinella* Hb in comparison with the relative unstability of *Riftia* HBL Hb (Terwilliger et al., 1980; Zal et al., 1996b), and they postulated that *Alvinella* Hb might possess structural adaptations to the extreme temperatures of its environment. Indeed, the optical density spectra of

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[1] Abbreviations: Hb, hemoglobin; HBL, hexagonal bilayer; DTT, dithiothreitol; BTP, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ESI-MS, electrospray ionization mass spectrometry; MaxEnt, maximum entropy; *m*/*z*, mass vs charge; MALLS, multiangle laser light scattering; FPLC, fast protein liquid chromatography; LMW, low molecular weight; HMW, high molecular weight; TEM, transmission electron microscopy.

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this Hb revealed only slight methemoglobin formation and it was very stable since no evidence of dissociation were found except at 50 °C (Terwilliger & Terwilliger, 1984).

In addition, *Alvinella* HBL Hb possesses a very high O_2 affinity in comparison with other species (0.20 Torr < P_{50} < 0.66 Torr, *in vitro*: diluted Hb, $T = 20$ °C, pH 7.6−7.0, 1 atm) (Toulmond et al., 1990). These authors also reported that at low temperature (10 °C) and high pH (7.6), the O_2 affinity of *Alvinella* Hb was so high (P_{50} lower than 0.1 Torr) that it could not be precisely measured with the Hemox technique.

In the present work we provide detailed data on the masses and complete polypeptide chain composition of *Alvinella* HBL Hb as determined by a very accurate technique of electrospray ionization mass spectrometry (ESI-MS) coupled with maximum entropy analysis (Ferrige et al., 1991, 1992). In addition, using the stoichiometry of the polypeptide chains and subunits constituting this Hb, and an accurate mass measurement of native HBL Hb obtained by multiangle laser light scattering (MALLS), we propose two alternative models of its quaternary structure.

EXPERIMENTAL PROCEDURES

Animal Collection and Sample Preparation

Alvinella used in this study were collected from 2600 m depth at 13°N site (12°46′N−103°56′W and 12°50′N−103°57′W) on the East-Pacific Rise during the HERO'92 expedition in April 1992 (A.T. and F.Z.). Worms were grasped using the manipulator of the American submersible Alvin and maintained at deep-sea water temperature (2−4 °C) in a thermally insulated container during the trip to the surface (2−3 h). On board the animals which had not been damaged during collection were dissected dorsally, and the blood, uncontaminated with coelomic fluid, was withdrawn from the main vessel into glass micropipettes and pooled on melting ice. The blood was centrifuged at low speed a few minutes, and the supernatant was frozen in liquid nitrogen until the purification step.

In the laboratory, the thawed sample was centrifuged 10 min at 10000 rev/min at 4 °C. Hb solutions were purified by gel filtration on a 1 × 30 cm Superose 6-C column (Pharmacia LKB Biotechnology, Inc.) using a low-pressure FPLC system (Pharmacia). The column was equilibrated with the following saline buffer: BTP 50 mM, 400 mM NaCl, 3 mM KCl, 32 mM $MgSO_4$, and 11 mM $CaCl_2$ (pH 7,0). Flow rate was typically 0.5 mL/min, and the eluate was monitored with an UV detector (Pharmacia). The elution product corresponding to the HBL Hb was collected and concentrated with a 10 kDa cutoff microconcentrator (Centricon-10; Amicon). One or two further purifications using the same protocol were performed to obtain clear fractions when necessary. The column was calibrated with the following marker proteins (HMW, Pharmacia): aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa).

Multiangle Laser Light Scattering

MALLS measurements were performed with a DAWN DSP system (Wyatt Technology Corp.) directly on-line with the FPLC system described above. The eluate was simultaneously monitored with an UV detector and a refractometer (Erma, 7512). The Debye fit method was used for molecular mass determination (Wyatt, 1993). In this method the variation rate of the refractive index as a function of concentration, d*n*/d*c*, was set as 0.190 mL/g, typical for proteins without glycan.

SDS−PAGE

SDS−PAGE was carried out on *Alvinella* HBL Hb using the discontinuous buffer system of Laemmli on slab gels (Laemmli, 1970). The gel dimensions were 1.5 mm × 16 cm × 18 cm (LKB instrument). The stacking gel was constituted of 4% and 2.7% of acrylamide and bisacrylamide, pH = 6.8, and the resolving gel by 10% and 2.7%, pH = 8.8. Dialyzed sample of Hb (1 mg/ml) were incubated at 100 °C for 90 s in the presence or absence of 5% 2-mercaptoethanol. Sample migration was performed at constant current of 150 mA using a Tris-glycine buffer, pH = 8.3, during about 2 h. Identification of subunit relationships after 2-mercaptoethanol treatment was done by two-dimensional electrophoresis process. A gel slice corresponding to the observed bands after SDS migration was cut and incubated under reducing conditions. It was then placed on top of a new gel and subjected to electrophoresis as described above. The gels were stained overnight on agitator in 0.125% Coomassie Brillant Blue R-250 (Bio-Rad) in 50% methanol, 10% acetic acid, and water. The molecular mass of each constituent polypeptide chain was determined using LMW protein standards (Pharmacia). The gels were scanned at 550 nm on a Vernon densitometer and the peaks area were determined by a Coradi planimeter.

Electrospray Ionization Mass Spectrometry

Electrospray data were acquired on a Quattro II mass spectrometer (Micromass UK Ltd.) scanning over the *m*/*z* range 600−2500 in 10 s/scan. About 45 scans were averaged to produce the final spectrum. Sample concentrations of 0.5 μg/μL in 50/50 acetonitrile/water containing 0.2% formic acid were introduced into the electrospray source at 5 μL/min. In order to establish the relationships between the subunits and their constituent polypeptide chains, a solution of the native Hb (5 μg/μL) was reduced with 10 mM DTT at room temperature (20 °C) and pH 8−9 (adjusted with ammonium bicarbonate). Incubation was allowed to proceed for typically 1, 5, 10, and 20 min, after which times 10 μL aliquots of the Hb solution were diluted with 90 μL of 50% aqueous acetonitrile containing 0.2% formic acid and analyzed by ESI-MS. Carbamidomethylation with iodoacetamide was carried out on the native and reduced Hbs for free and total cysteine (Cys) determination, respectively, under the same conditions as carboxymethylation (Crestfield et al., 1963). Deglycosylation was carried out using recombinant *N*-glycosidase F (Boehringer Mannheim Corp.) (Tarentino et al., 1985). Mass scale calibration employed the multiply-charged series from horse heart myoglobin (M_r = 16951.5 Da; Sigma catalog no. M-1882). Molecular masses are based on the atomic weights of the elements (IUPAC, 1993). The raw ESI-MS spectra were processed using a MaxEnt based approach (Ferrige et al., 1991, 1992) employing the MemSys5 program (MaxEnt Solutions Ltd., Cam-

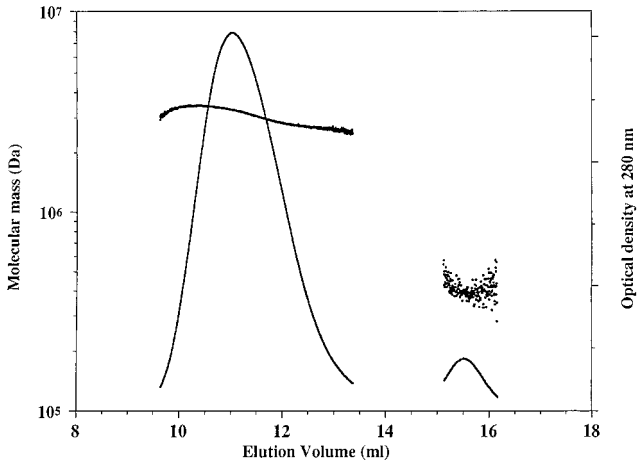

FIGURE 1: Gel filtration separation and MALLS mass measurement of *A. pompejana*'s Hb. Optical density at 280 nm (lines) and molecular mass (Da; dots) versus elution volume (mL). The slope of mass estimates along the elution profile indicates the mass polydispersity.

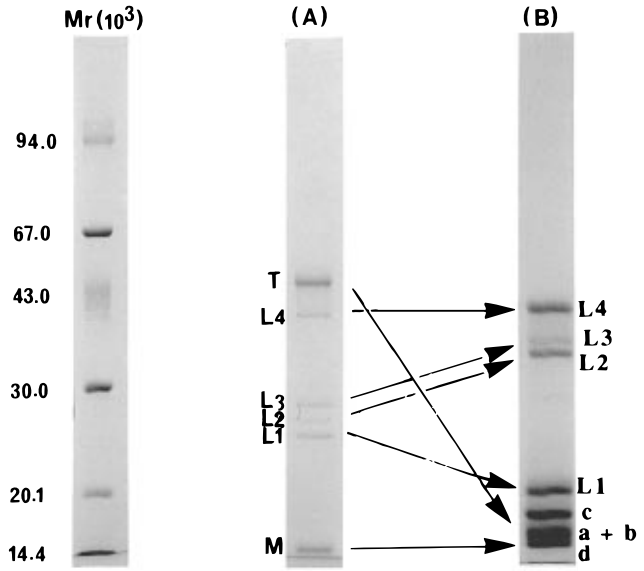

FIGURE 2: SDS−PAGE of *A. pompejana*'s Hb. Lane (A), unreduced Hb; lane (B), after reduction with mercaptoethanol. The approximate M_r were determined by LMW protein calibrates from Pharmacia (left lane).

bridge, U.K.) incorporated as part of the VG MassLynx software suite.

RESULTS

Hb Molecular Mass Determined by FPLC and MALLS

Samples of *A. pompejana* Hb generally showed two major symmetrical peaks in gel filtration (Figure 1). In addition, several minor peaks can be observed after the peak 2. These fractions were not collected and could correspond to the dissociation products from Hb. The apparent M_r of the first and second peaks of *Alvinella* Hb, estimated using HMW markers, corresponded to about 3600 kDa and 400 kDa. Molecular masses estimated by MALLS analysis during the whole elution of the peaks (Figure 1) yielded more accurate results for *Alvinella* Hb fractions: 3833 ± 14 kDa ($n = 841$) for the first peak and 331.9 ± 24 kDa ($n = 250$) for the second.

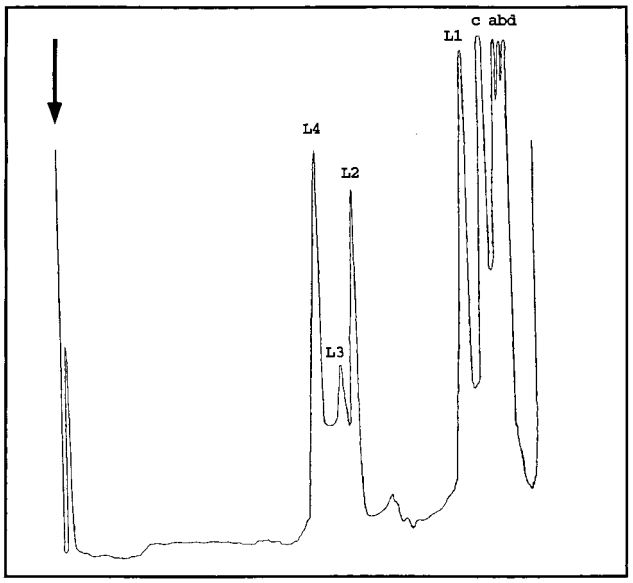

FIGURE 3: Densitometric profile at 550 nm of the gel obtained from Figure 2B. The top of the gel is indicated by the arrow and the direction of the electrophoresis is from left to right. The peaks are numbered in accordance with Figure 2B.

Polypeptide Chain Composition Determined by SDS−PAGE

Figure 2 presents the SDS−PAGE profiles of *Alvinella* Hb in the presence or absence of reducing agent. The unreduced Hb dissociated in SDS into six subunits (lane A): subunit *M* with an apparent molecular weight of 14 600, subunits *L1*, *L2*, and *L3* with M_rs 25 000, 26 000, and 27 000, subunit *L4* with M_r close to 38 000, and subunit *T* with M_r 50 000. After reduction (lane B), *Alvinella* Hb dissociated into eight polypeptide chains consisting of two groups: one in the range M_r 14 000−21 000 (*a*, *b*, *c*, *d*, and *L1)* and the other in the range M_r 34 000−38 000 *(L2−L4).* Subunit *M* corresponds to the monomeric globin chain *d*, subunit *T* to a disulfide-bonded trimer of globin chains *a*, *b*, and *c*, and subunits *L1−L4* correspond to the monomeric linker chains. Figure 3 shows the densitometric profile of the gel presented on Figure 2. On this scan, the percentage of each peak corresponds to *L4* (15.05%), *L3* (3.77%), *L2* (9.54%), *L1* (18.23%), *c* (20.47%), and *a*, *b*, *d* (32.94%), resulting in a globin:linker ratio of 53.4:46.6. Several scans of reduced *Alvinella* Hbs were performed, giving an average proportion of 47 ± 4% ($n = 5$) for the linker chains.

Polypeptide Chain Composition Determined by ESI-MS

The subunits and polypeptide chains are named according to the nomenclature used for *Lumbricus terrestris* HBL Hb (Fushitani & Riggs, 1988). Note that, although we have used the same nomenclature in SDS−PAGE and ESI-MS results, the assignments of some of these chains are probably different between the two techniques, particularly for chains *a* and *d*. Figure 4A shows the spectrum obtained from native *Alvinella* Hb after deconvoluting the raw ESI data by the MaxEnt program, revealing four monomer chain (*a1−a4*), three major and one minor linker chains (*L1−L4*) and a trimer (*T*). Their molecular weights are summarized in Table 1. Furthermore, in some ESI-MS experiments chains *L1*, *L2*, and *L4* appear to possess a heme group as indicated by peaks corresponding exactly to the mass of a given

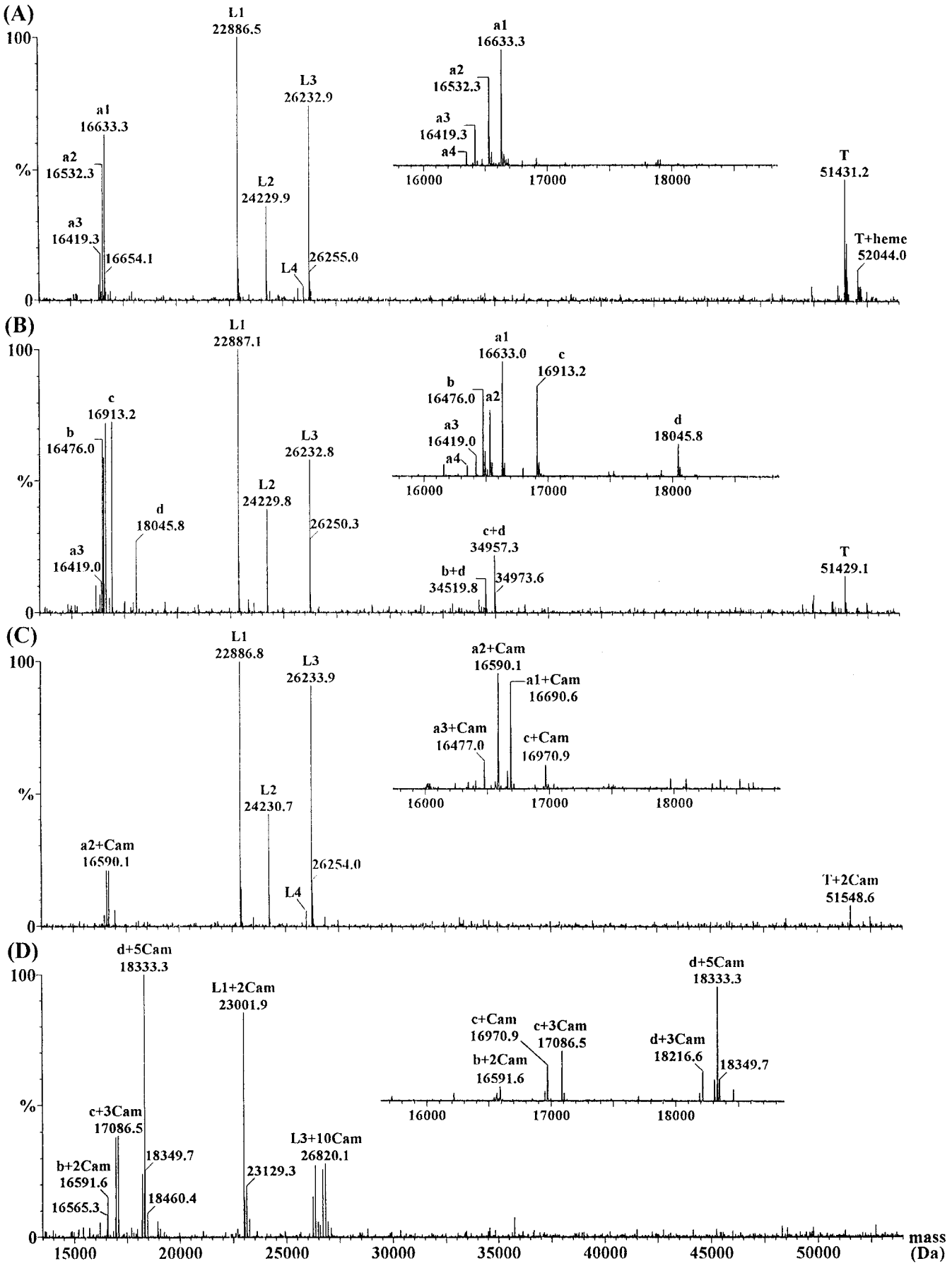

FIGURE 4: MaxEnt processed ESI spectra of *A. pompejana* Hb. (A) Native Hb. (B) Reduced Hb (5 mM DTT for 10 min). (C) Carbamidomethylated Hb. (D) Reduced (10 mM DTT for 5 min) and carbamidomethylated Hb. The insets show the region of the globin chains on an expanded scale.

Table 1: Summary of ESI-MS Results of *A. pompejana* HBL Hb Subunits and Polypeptide Chains

chain/subunit	mean native mass (Da)[a]	rel int.[b]	Cam mass (Da)[c]	free Cys[d]	reduced mass (Da)[e]	red/Cam mass (Da) [f]	corrected mass (Da)[g]	total Cys[h]
monomers								
a4	16348.9 ± 1.0	0.09	16406.8 ± 1.0	1	16348.7 ± 1.5	ND[j]	ND	ND
a3	16419.6 ± 1.0	0.24	16476.3 ± 1.0	1	16418.9 ± 1.5	ND	ND	ND
a2	16532.4 ± 1.0	0.80	16589.7 ± 1.0	1	16532.0 ± 1.5	16706.1 ± 1.0	16534.9 ± 1.0	3
a1	16633.4 ± 1.0	1.00	16630.6 ± 1.0	1	16633.0 ± 1.5	16807.3 ± 1.0	16636.1 ± 1.0	3
trimer *T*[i]	51431.9 ± 4.0		51548.6 ± 4.0	2	ND	ND		
b		0.65			16476.3 ± 1.0	16591.6 ± 1.0	16477.5 ± 1.0	2
c		1.00			16913.1 ± 1.0	17087.3 ± 1.0	16916.1 ± 1.0	3
d		0.61			18044.6 ± 1.0	18334.1 ± 1.0	18048.8 ± 1.0	5
linkers								
L1	22887.1 ± 2.0	1.00	22886.5 ± 2.0	0	22887.2 ± 2.5	23003.0 ± 2.0	22888.9 ± 2.0	2
L2	24230.5 ± 2.0	0.36	24230.3 ± 2.0	0	24229.8 ± 2.5	24808.6 ± 3.0	24238.1 ± 3.0	10
L3	26233.6 ± 2.0	0.73	26233.6 ± 2.0	0	26232.9 ± 2.5	26818.4 ± 3.0	26247.9 ± 3.0	10
L4	25974.4 ± 2.0	0.05	25975.2 ± 2.0	0	ND	ND	ND	ND

[a] Mean of five determinations on native Hb ± estimated error. [b] Intensity relative to most intense peak in each group in native condition for the polypeptide chains *a* and *L*, and after reduction for the three trimer components (*b*, *c*, and *d*). [c] Mean of four determinations on carbamidomethylated Hb ± estimated error. [d] Number of free Cys in native chain or subunit derived by dividing (Cam mass − native mass) by 57 and rounding to nearest integer. [e] Mean of two determinations on reduced Hb ± estimated error. [f] Mean of seven determinations (two determinations for *L2* and *L3*) on reduced/carbamidomethylated Hb ± estimated error. [g] Red/Cam mass corrected for carbamidomethylation (57.052 Da/Cys). Values are reduced masses ± estimated error. [h] Total Cys in chain derived by dividing (Red/Cam mass − reduced mass) by 57 and rounding to nearest integer. [i] Composed of chains *b*+*c*+*d*. Note that the sum of corrected masses for *b*+*c*+*d*-8H (for two intra- and two interchain disulfide bonds) is 51434.3 Da. [j] ND, not determined.

Table 2: Heme Intensities in Function of the Linker Intensities As Observed by ESI-MS on Several HBL Hbs[a]

species	linker	% with heme
Alvinella pompejana		
"native condition"	L1	(1.1−2.3) ± 1.2
	L2	ND[b] - 3.1
	L3	ND - 3.5 ± 0.5
Tylorrynchus heterochaetus		
"native condition"	linker dimer (2*L2*)	4.0 ± 1.2
"reduced condition"	L2	4.7
Nereis diversicolor		
"native condition"	linker dimer	4.3
Arenicola marina		
"native condition"	linker dimer	5.3

[a] Note: These observations were made by B.N.G. [b] ND, not detected.

polypeptide chains plus one heme group (Table 2). However, these adducts appear at a very low level, *i.e.*, 1.1% < heme < 3.5% of the corresponding linker chain intensity, an may not reflect a native condition. The MaxEnt deconvoluted spectrum of the Hb after partial reduction with DTT (Figure 4B) shows that *T* has significantly decreased in intensity and three new components (*b*, *c* and *d*) have appeared. Their masses are also given in Table 1. The sum of the masses of these new components is close to the mass of the trimer, clearly indicating that *T* is a covalent trimer of *b*, *c*, and *d*. Furthermore, the occurrence of two peaks corresponding in mass to (*b*+*d*) and (*c*+*d*) indicates that chain *d* is linked to both chains *b* and *c* in the trimer. The second FPLC peak (Figure 1) was also analyzed by ESI-MS in native condition and was found to contain only the monomeric globin chains *a* and the trimer *T*, suggesting that this 330 kDa Hb fraction is a dissociation product of the HBL Hb or another population of molecules (Figure 5).

Cysteine Residue and Carbohydrate Content

The number of free Cys and disulfide bonds in each chain was determined by comparing the masses determined from the reduced or native masses with those obtained after carbamidomethylation with and without reduction. Figure 4C is the MaxEnt deconvoluted spectrum obtained after carbamidomethylation of the native Hb without reduction and shows that the monomeric chains (*a1*−*a4*) and the trimer *T* possess one and two free Cys, respectively. This is inferred from the addition of one and two carbamidomethyl groups to the native components (57.052 Da/Cys), respectively. The masses of *L1*−*L4* remained the same, implying that these chains contain no free Cys.

Figure 4D presents the spectrum obtained after reduction and carbamidomethylation of *Alvinella* Hb and shows that the monomeric chains *a1* and *a2* each contain 3 Cys and that chains *b*, *c*, and *d*, constituting the trimer *T*, possess 2, 3, and 5 Cys, respectively. These latter results, when combined with the information that the trimer has two free Cys and that chain *b* and *c* are both linked to chain *d*, are consistent with the structure of the trimer shown in Figure 6. Chains *a3* and *a4* were not detected in these data and hence their Cys content was not established.

The data in Figure 4D also infer that there are at least 10 Cys in linker chains *L2* and *L3* but apparently only two in *L1*. However, it may be that *L1* is particularly resistant to the reduction. The masses of the components measured from the carbamidomethylated samples are summarised in Table 1.

After deglycosylation, the masses of the subunits and the polypeptide chains remained unchanged, indicating that there is no glycoprotein in *Alvinella* Hb.

Table 3 presents the relative intensities of each peak, including all identified adducts which intensity was over 2.5%, for four different determinations.

DISCUSSION

Molecular Mass of Native Alvinella Hb

A knowledge of the accurate molecular mass of the native Hb is crucial for the calculation of the number of polypeptide chains involved in the hetero-multimeric complex. However, there is appreciable scattering in the published values for the M_r of HBL Hbs obtained with different techniques and

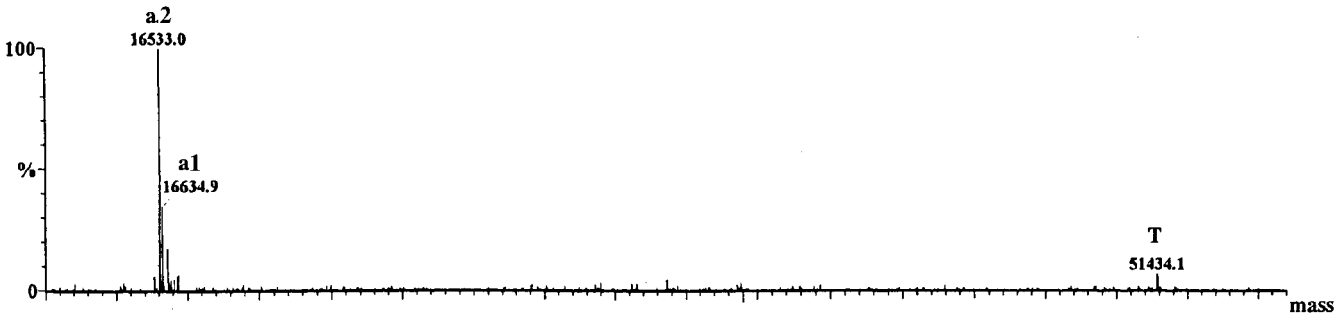

FIGURE 5: MaxEnt processed ESI-MS spectra of the second peak revealed by the Figure 1 in native condition.

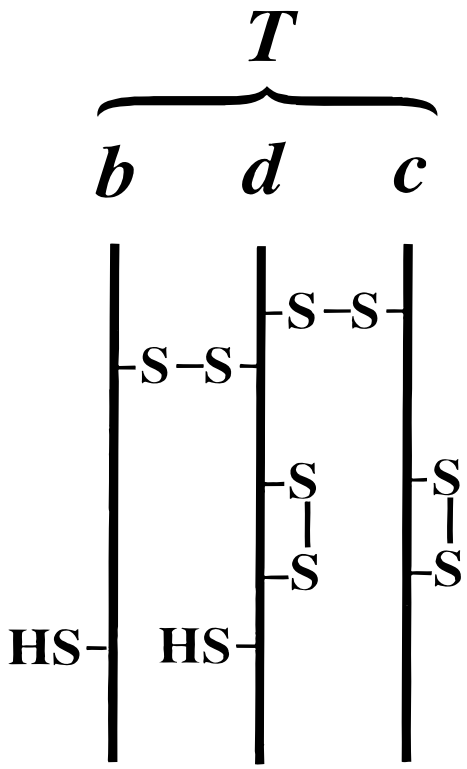

FIGURE 6: Schematic representation of Cys residues located on the globin chains *b*, *c*, and *d* which form the trimer *T* of *A. pompejana*'s HBL Hb based on data from Figure 4 and Table 1. Cys residues are either free (−SH) or involved in intra- or interchain disulfide bridges (−S−S−). Locations of the residues on the chains are arbitrary.

preparation conditions [reviewed in Vinogradov and Kolodziej (1988)]. The mass found by FPLC for *Alvinella* Hb is typical of the masses of other HBL Hbs (Lamy et al., 1996) and is also very close to the M_r reported in previous studies (*i.e.*, 3600 kDa; Terwilliger & Terwilliger, 1984; Toulmond et al., 1990). However, measurements obtained by FPLC are subject to variations because the migration depends mainly on the shape of the molecule and its interactions with the gel. We have recently been using MALLS as a *quasi* absolute method for determining the molecular mass of macromolecules since it does not require any "universal" calibration or other *a priori* assumptions (Wyatt, 1993; Zal et al., 1996b, 1997a). MALLS has the advantage that it can be performed directly on-line with separative techniques such as FPLC using a physiological buffer as eluent, thus avoiding sample manipulation and possible deterioration. The molecular mass found for *Alvinella* HBL Hb by MALLS was 3833 ± 14 kDa. This result is significantly higher than that reported earlier for this species (Terwilliger & Terwilliger, 1984; Toulmond et al., 1990) and falls in the upper range reported for other HBL Hbs (Vinogradov et al., 1982; Vinogradov, 1985a,b), even considering recent studies using MALLS measurements (Zal et al., 1996b, 1997a).

Table 3: Relative Intensities of Each Components Constituting *A. pompejana* HBL Hb[a]

chains/subunits	"native" condition		reduction	
			after 10 min	after 30 min
a1	21.46	16.85	85.87	89.67
a2	15.50	12.54	70.22	74.35
a3	3.68	3.73	17.65	16.46
a4	1.41	1.22	7.77	6.68
b	1.44	0.69	97.69	96.60
c	1.74	0.70	71.84	142.03
d	ND[b]	ND	44.59	81.30
L1	36.47	28.24	129.05	131.74
L2	11.16	9.23	66.53	63.38
L3	26.26	21.26	95.00	86.98
L4	1.44	1.08	ND	ND
(b+d)	ND	ND	20.47	ND
(d+c)	ND	ND	36.64	10.36
T	22.98	33.10	21.29	4.33

[a]Note: For all data set we have added the area of all peaks ≥2.5% threshold in the foregoing mass intervals of all major peaks. [b] ND, not detected.

SDS−PAGE Profiles of Alvinella Hb

SDS−PAGE profiles of *Alvinella* Hb resemble those found for *Lumbricus terrestris* (Vinogradov et al., 1980; Martin et al., 1996) and seem typical of annelid Hbs, as previously mentioned by Terwilliger and Terwilliger (1984) and Toulmond et al. (1990). Our results differ slightly from those of these authors, mainly in the number and masses of the linker chains. Indeed, Terwilliger and Terwilliger (1984) found only two bands with M_r 36 500 and 30 000, whereas Toulmond et al. (1990) resolved three bands with M_r 30 000, 28 000, and 25 000. We have no explanation for the difference between our results and those of these authors. However, the SDS−PAGE data profiles presented in this paper are very clear, showing very good resolution for such experiments. Further, the number of components resolved is supported by our ESI−MS results, naturally with mass differences but it is well-known that in some cases SDS−PAGE is not well adapted for accurate mass measurements (Reynolds et al., 1970; Fish et al., 1970), and especially with HBL Hbs (Vinogradov et al., 1980). In addition, the globin:linker ratio of 53:47 ($n = 5$) is surprisingly different from that usually found for other HBL Hbs (*i.e.*, 70:30) using the same method (Vinogradov et al., 1980; Vinogradov, 1985a,b). Two non-exclusive hypothesis could explain this result: (1) *Alvinella*'s HBL Hb contains more linker chains than other

Table 4: Number of Copies of Each Component Constituting *A. pompejana* HBL Hb Using the Data Presented in Table 3[a]

chains/subunits	"native" condition		reduction		mean	SD
			after 10 min	after 30 min		
$\sum a$	42.05	34.34	40.62	43.98	40.25	4.17
$\sum b$	24.42	33.79	31.20	23.72	28.28	4.98
$\sum c$	24.72	33.80	29.04	36.82	31.09	5.32
$\sum d$	22.98	33.10	27.52	22.55	26.53	4.91
$\sum L$	75.33	59.81	65.02	66.29	66.61	6.45
$\sum G$	114.17	135.03	128.38	127.07	126.16	8.72
$\sum L$	75.33	59.81	65.02	66.29	66.61	6.45
total	189.50	194.84	193.40	193.36	192.77	15.17
$\sum b + \sum c + \sum d$	72.12	100.69	87.76	83.09	85.90	15.21
ratio *G*	0.60	0.69	0.66	0.65	0.65	0.04
ratio *L*	0.40	0.31	0.34	0.35	0.35	0.04

[a] Note each component were calculated assuming that the ionization behavior of each polypeptide chain was the same and gave a sensitivity factor *K* ($K = M_r$ HBL Hb/$\sum I_i M_{r_i}$), where M_{r_i} and I_i are the M_r of the polypeptide chain *i* and the relative intensity, respectively. Afterward, the number of copies of each component, N_i, is calculated using the following equation: $N_i = KI_i$.

Table 5: Models for the Quaternary Structure of *A. pompejana* HBL Hb

subunit	chain	mass[a]	copies using experimental M_r[b]	SD[c]	copies in model 1[d]	total mass of model 1	copies in model 2[d]	total mass of model 2
monomers	$\sum a$	17180.7	40.25	4.17	36	618505.2	48	824673.6
	b	17093.0	28.28	4.98	36	615348.0	24	410232.0
	c	17529.6	31.09	5.32	36	631065.6	24	420710.4
	d	18661.3	26.53	4.91	36	671806.8	24	447871.2
total globin linkers					144	2536725.6	120	2103487.2
	$\sum L$	24290.3	66.61	6.45	60	1457420.4	72	1748904.5
total mass of Hb						3994146.0		3852391.7
experimental mass[e]						3833000.0		3833000.0

[a] Masses from Table 1 including heme group except for $\sum a$ and $\sum L$ that correspond to the mean mass of the polypeptide chains *a* and *L*, respectively. [b] Number of copies for each chain calculated using the mass of the whole Hb determined by MALLS (*cf.* Table 4). [c] Standard deviation for the number of copies of each chain (*cf.* Table 4). [d] Number of copies proposed in our models for each chain, based on the column labeled "copies using experimental M_r", and constrained for the assumed D_6 symmetry of the whole molecule. [e] Value obtained by MALLS.

HBL Hbs so far investigated; (2) *Alvinella*'s linker chains bind more Coomassie reagent for an unknown reason. Consequently we did not attempt to build a model of HBL Hb quaternary structure from SDS−PAGE data. However, the recent studies using ESI-MS to characterize the primary composition of several extracellular Hbs (Green et al., 1995; Weber et al., 1995; Martin et al., 1996; Yuasa et al., 1996; Zal et al., 1996a, 1997a) have demonstrated that materially more detail can be provided by this technique than by SDS−PAGE. In addition to giving unambiguous and precise values for the molecular weight (<±0.01%), ESI-MS−MaxEnt offers substantially higher resolution than SDS−PAGE by having the ability to resolve two proteins separated by as little as 6 Da at 16 kDa. Hence, it is becoming the method of choice for establishing realistic models of complex macromolecules.

Model of Alvinella HBL Hb Quaternary Structure

MaxEnt analysis of ESI-MS spectra produces semiquantitative 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 relative intensities presented in Table 3, an estimation of the number of copies of each chain in the whole molecule can be derived (Table 4) assuming that each chain behave similarly, *i.e.*, that the ratio $K =$ (peak intensity)/(number of copies) is the same for each component. Knowing the mass of *Alvinella*'s Hb as obtained by MALLS and the exact masses of each chains as determined by ESI-MS, and further assuming that the molecule possesses a D_6 point-group of symmetry, as evidenced by TEM (Terwilliger & Terwilliger, 1984) and 3-D reconstruction by cryo-microscopy (de Haas et al., 1996), we propose two alternative models of the *Alvinella* Hb quaternary structure (Table 5).

Table 6: Comparison of Globin:Linker Ratios in Percentage Obtained by Three Different Techniques

species	HPLC[a]	SDS−PAGE[b]	ESI-MS[c]	references
M. decora	72:28	70:30	71:29	Weber et al., 1995
L. terrestris	73:27	ND	78:22	Martin et al., 1996
T. heterochaetus	ND[e]	ND	75:25	Green et al., 1995
R. pachyptila	70:30	71:29	75:25	Zal et al., 1996a
A. marina	72:28	73:27	71:29	Zal et al., 1997a
A. pompejana[d]	ND	53:47	65:35	this study

[a] High performance liquid chromatography. [b] Sodium dodecyl sulfate−polyacrylamid gel electrophoresis [c] Elecrospray ionization mass spectrometry. [d] For the ESI-MS ratio we have used the mean of four BPI determinations (2 native and two reduced experiments, *cf.* Table 4) including the area of all peaks ≥2.5% threshold in the foregoing mass intervals of all major peaks. [e] ND, not determined.

In the first model, the whole molecule would consist of 144 globin-like chains (36 monomers and 36 trimers) and 60 linker chains. This model gives a number of globin:linker chain ratio of 70:30 (*i.e.*, 144/204:60/204), in good agreement with the ratio of other HBL Hb but outside the ESI-MS data (65 ± 4:$35 \pm 4 = 126/192$:$66/192$) or SDS−PAGE experiments (53:47) (Tables 4 and 6). The one-twelfth protomer of *Alvinella* Hb will be composed on the bracelet model scheme, 3 monomeric chains *a* and 3 trimer *T*,

$[(a)_3T_3]$, with a calculated molecular mass of 211 kDa, including heme, this association corresponding to a dodecamer. The native Hb would be built by the association of 12 dodecamers plus the 60 linker chains yielding for the whole molecule a calculated molecular mass of 3995 kDa, 4.2% higher that native molecule as determined by MALLS (3833 kDa).

In the second model, the whole molecule would consist of 120 globin-like chains (48 monomers and 24 trimers) and 72 linkers chains. This model provides a number of globin: linker chain ratio of 62:38 (*i.e.*, 120/192:72/192) very close of ESI-MS data (Table 4). In this model the one-twelfth protomer would be made by the association of 4 monomers and 2 trimers, $[(a)_4T_2]$, with a calculated molecular mass of 175 kDa, including heme group. The native Hb would be built by the assembly of 12 decamers plus 72 linkers chains providing a M_r of 3853 kDa, only 0.5% higher that MALLS results (accuracy ±2%). In this model, the second Hb fraction isolated during the purification step could correspond to a pair of such decamers, $[(a)_4T_2]_2$, giving a M_r around 350 kDa (including heme) close to the experimental value of 331.9 ± 24 kDa found by MALLS. To form the native molecule, six pairs of protomers (decamer) would be arranged around a large central structure made of linker chains, in such a way that the whole structure would resemble other HBL Hbs, as indicated by TEM micrographs of the native molecule (Terwilliger & Terwilliger, 1984; Toulmond et al., 1990) and by 3-D reconstruction by cryo-microscopy (de Haas et al., 1996). However, the 3-D reconstruction of *Alvinella*'s HBL Hb showed clearly a local pseudo 3-fold symmetry at the protomer level called hollow globular substructures (de Haas et al., 1996). This symmetry, which is not in agreement with the existence of decamer protomer (*i.e.*, model 2), fits well with the dodecamer concept (*i.e.*, model 1). Nevertheless, incidentally we can also note that a nonamer possess a 3-fold symmetry closer to a decamer than a dodecamer.

Unusual Properties of Alvinella HBL Hb Revealed by ESI-MS

Data derived from ESI-MS analysis establish that *Alvinella* Hb is composed of (i) monomeric globin chains, (ii) a disulfide-bonded trimer of globin chains, and (iii) four linker chains. This pattern, specifically the existence of a trimer, clearly puts *Alvinella* Hb in the polychaete/oligochaete subgroup as opposed to the achaete/vestimentiferan subgroup which possesses dimers instead of trimers (Lamy et al., 1996). However, a number of differences render *A. pompejana* HBL Hb quite unusual and set it apart from all other HBL Hbs studied so far, including closely related monomeric globin-like chains, a large number of globin-like chains with free Cys, and a high proportion of linker chains.

Monomeric Globin-Like Chains

First, the ESI mass spectrum revealed four monomeric globin-like chains, *a1*−*a4*, the mass differences between which suggest that *a2*−*a4* may be derived from *a1*. Indeed, the mass differences $a1 - a2 = 101.0$ Da, $a2 - a3 = 112.8$ Da, and $a3 - a4 = 70.7$ Da, are remarkably close to the masses of the amino acid residues T (101.1), I or L, (113.2), and A (71.1), respectively. It is unlikely that these components are produced by the mass spectrometer since the experimental conditions used are unable to cleave peptide bonds. Two hypotheses may be suggested: (i) there is only one gene, and the different chains observed are due to post-translational events or proteolytic degradations during sample preparation; (ii) there are actually four different chains in the native molecule. Further studies of the primary sequence of these proteins and its/their gene(s) are needed to resolve this point.

Cysteine Contents and Sulfide Binding

Second, in *Alvinella* Hb the polypeptide chains *b* and *d*, implicated with chain *c* in the trimer *T*, contain two and five Cys residues respectively, with one free Cys on each chain (Figure 4). There is also one free Cys on the monomeric chains *a1*−*a4* (Table 1). Annelid and tube-worm globin-like chains have been classified into four categories according to their Cys content: all four groups possess an intrachain disulfide bond, and three of these groups contain a supplementary Cys, at different positions, participating in an interchain disulfide bond (Suzuki et al., 1988a, 1989; Fushitani et al., 1988). Clearly, while some *Alvinella* Hb globin chains possess inter- and intrachain disulfide bonds, the existence of free Cys in most chains seems atypical and *Alvinella* does not fall easily into the classification scheme described above. Moreover, for chain *b*, our data imply that it possesses two Cys residues, one free and one involved in an interchain disulfide bond with chain *d* (Table 1; Figure 6), thus making it the first globin from an annelid HBL Hb lacking an intrachain disulfide bridge.

In recent studies, free Cys have been found in a number of globin chains from annelid or tube-worm Hbs. In the vestimentiferans *Lamellibrachia* and *Riftia*, some globin chains possess free Cys (Suzuki et al., 1989, 1990a; Zal et al., 1996a, 1997b). Similarly, there is a free Cys in the monomeric chains *a1* and *a2*, and in the polypeptide chain *d* involved in the trimer of *Arenicola marina* Hb (Zal et al., 1997a), and also in the monomeric globin chains *a1*−*a5* and the globin chains *b* and *c*, involved in the dimer of *Oligobrachia mashikoi* Hb, a pogonophoran worm (Yuasa et al., 1996). Similar observation was recently reported for the monomeric globin chain from the earthworm *Pontodrilus matsushimensis* (Shishikura & Nakamura, 1996). These free Cys may be able to bind sulfide, either for detoxification purposes (*i.e.*, sulfide immobilization by Hb) as suggested for *Arenicola* (Zal et al., 1997a), or for sulfide transport as an adaptation to symbiotic life as in vestimentiferan (Arp et al., 1987; Zal et al., 1997b) or in pogonophoran, in which sulfide binding is strongly probable (Terwilliger et al., 1987). Indeed, the presence of free Cys in HBL Hbs seems to be a characteristic of species living in sulfide rich environments (up to several hundred μmol L^{-1}) such as all the worms mentioned above. Although no indication of sulfide binding by *Alvinella* Hb has been published, this possibility does not seem unlikely. Jouin-Toulmond et al. (1996) observed numerous dark granules, which could correspond to sulfide accumulation, in the coelomic walls of the ventral vessel and of the efferent vessels coming from the gills. They suggest that sulfide could penetrate through the branchial surface and be bound by Hb, a further detoxification process operating in the coelomic wall of these anterior blood vessels.

The Roles of Linker Chains in HBL Hbs

One of the major results highlighted by ESI-MS analysis of *Alvinella* Hb is the high number of linker chains (66.1 ± 6.4 by ESI-MS, Table 4, and either 60 or 72 in the proposed models, Table 5) in comparison with other HBL Hbs (Vinogradov 1985b), even those already investigated by ESI-MS (Table 6). This proportion seems to be a characteristic of alvinellid HBL Hbs since it was also observed in *Alvinella caudata*, *Paralvinella grasslei*, and *Paralvinella palmiformis* (F.Z., unpublished observations). Furthermore, some of the alvinellid linker chains seem to be resistant to reduction: they are not destroyed by DTT treatment unlike those of the achaete annelid *Macrobdella decora* (Weber et al., 1995), the polychaete annelids *T. heterochaetus* (Green et al., 1995) and *A. marina* (Zal et al., 1997a), the oligochaete annelid *L. terrestris* (Martin et al., 1996) or the vestimentiferan *Riftia pachyptila* (Zal et al., 1996a). This particularity probably explains the difficulty we had in carbamidomethylation experiments. Indeed, it seems unlikely that *L1* possesses only two Cys because in all HBL Hbs investigated to date, linker chains possessed at least ten Cys residues.

Moreover, on some ESI-MS experiments three of *Alvinella* linker chains were observed with heme adducts (*L1*, *L2*, and *L3*, Table 2). However, we do not know if this low level of heme adduction reflects a native condition or if it is merely an artefact due to the sample preparation as observed for other HBL Hbs by one of us (B.N.G. observations, Table 2). Further investigations will be necessary to verify this important point. However, these observation raise an important question about the precise role of the so-called linker chains in HBL Hbs, especially if they bind heme as suggested by ESI-MS: Are they limited to a structural role or do they play other functions?

There is little doubt that the specific architecture of HBL Hbs is only possible through the existence of these linker chains, as originally proposed by Vinogradov et al. (1982). For example, *Lumbricus* Hb preparations deficient in or lacking linker chains do not form the whole HBL Hb molecule (Kapp et al., 1987; Suzuki et al., 1988b; Vinogradov et al., 1986, 1991; Fushitani & Riggs, 1991). Likewise, vestimentiferan tube-worms possess a multihemoglobin system with three different extracellular Hbs, two of them dissolved in the vascular blood (V1 and V2) and one in the coelomic fluid (C1). While V1 Hb possesses linker chains and shows a quaternary structure typical of HBL Hbs, the smaller Hbs V2 and C1 ($M_r \approx 400$ kDa) are devoid of linker chains and, although they contain roughly the same globin chains as V1, they do not form HBL structures (Suzuki et al., 1989; Zal et al., 1996a,b). An exception to this rule is the HBL Hb of the polychaete *Travisia japonica* which reportedly consists of only five polypeptide chains containing heme with molecular weights in the range 14 000−18 000 Da (Fushitani et al., 1982). In *Alvinella* Hb, considering the potentially high temperatures that this species may encounter (Chevaldonné et al., 1992), the high proportion of linker chains may explain the thermostability of the molecule. A few other polychaetes, *e.g.*, the hesionid *Hesiolyra bergi* (Blake, 1985) or the polynoid *Lepidonotopodium* sp. (Hourdez, personal communication), are found within *Alvinella* colonies, and the examination of their HBL Hbs could allow this hypothesis to be tested.

Beside their evident contribution to the structure of the molecule, other roles have been tentatively ascribed to the linker chains of HBL Hbs. They may contribute to the Hb specific buffer power as suggested by Toulmond (1979) or exhibit a methemoglobin reductase activity (Ascoli et al., 1978). More recently, Zal et al. (1997b) suggested a possible functional role for linker chains in sulfide binding in *Riftia* V1 HBL Hb, on the basis of their high Cys content, as determined by ESI-MS, and on sulfide-binding experiments. Also, in *Lumbricus*, Gibson et al. (1991) demonstrated an ability of isolated linker chains to bind CO and NO, thus substantiating the existence of a functional heme pocket in some of these supposedly structural components. Indeed, *Alvinella* would not be the only species possessing heme-containing linker chains in its Hb. The linker chain *L2* of *Lumbricus* Hb also binds heme (Mainwaring et al., 1986; Ownby et al., 1993; Martin et al., 1996).

The possibility for linker chains to bind heme is not completely surprising when their evolutionary origin is considered. Suzuki and Riggs (1993) showed that linker chain *L1* from the earthworm *Lumbricus* has evolved by fusion of two heme-binding genes to form a two-domain gene, followed by the loss of the first exon in domain 1 and the last exon of domain 2. The amino acid sequence of linker chains *L1* from *Lumbricus* and *AV-1* from *Lamellibrachia* (Suzuki et al., 1990b) revealed that domain 1 retained the heme-binding proximal histidine, but not domain 2. In addition, *L1* from *Lumbricus* also retained the distal histidine on domain 1 (Suzuki & Riggs, 1993).

In *Lumbricus* HBL Hb, the percentage of linker chains binding heme is between 34.7%, as revealed by HPLC (Ownby et al., 1993), and 16.6%, according to ESI-MS results (Martin et al., 1996). In *Alvinella*, if the linker chains (*L1*, *L2*, and *L3*) bind heme, considering their overall proportion in comparison with other HBL Hbs, this could help maintain a high oxygen-binding capacity for the whole molecule. A possible implication of linker chains in the adaptation of *Alvinella* Hb oxygenation properties to the extreme environment where it lives should certainly be considered. For example, the oxygenation properties of these three chains, yet to be studied, could explain the extremely high oxygen affinity of *Alvinella* HBL Hb even at high temperature.

In conclusion, in *Alvinella*, linker chains could fulfil three specific needs for this species, namely thermostability, oxygen-binding capacity, and sulfide trapping, but direct evidence is needed to support these hypotheses. Although *Alvinella* and its Hb may be exceptional in several ways, in relation to its extreme environment, we believe that its peculiar features may shed new light on the architecture and functioning of HBL Hbs in general.

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