Recent New Characterizations on the Giant Extracellular Hemoglobin of *Glossoscolex paulistus* and Some Other Giant Hemoglobins from Different Worms

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1. Introduction

Giant extracellular hemoglobins, also known as erythrocruorins, have been investigated as a model of extreme complexity in oxygen-binding heme proteins [1,2]. They are characterized by a very high molecular mass (*MM*) of several megadalton (MDa), and their oligomeric structure together with the crowded and protected heme environment are two of the main factors responsible for the high redox stability [3,4]. Superoxide dimustase (SOD)-like intrinsic activity, observed for hemoglobins of *Lumbricus terrestris* (HbLt) and of *Arenicola marina* (HbAm), is another important factor [5,6]. These hemoglobins present a highly cooperative oxygen binding and a peculiar behavior associated to their oligomeric dissociation into smaller subunits and possible rearrangement back into the native oligomeric structure [7,8].

Moreover, a strong motivation to study these giant hemoglobins is related to their potential use in medicine as blood substitutes. Studies have been performed in the past for HbLt [9], and are presently underway to test and validate the use of HbAm in this direction [6,10]. They seem to be very promising due to the lack of undesirable immunological reactions in tests with animals, explained by the absence of cell membranes as occurs with human hemoglobin in red blood cells [6,10]. Besides, the resistance to oxidation of extracellular hemoglobins, as noticed by their redox stability, is also an advantage as compared to the use of human hemoglobin in this medical application.

The giant extracellular hemoglobin of *Glossoscolex paulistus* (HbGp) is similar to several homologous proteins described in the literature [11-14]. These extracellular hemoglobins are constituted by a large number of globin-like subunits containing heme groups with *MM* in the range 15–19 kDa. These globin subunits form monomers of 16 kDa (*d*) and disulphide bound hetero-trimers of 51-52 kDa (*abc*), linked by non-heme structures (24–32 kDa), named linkers (*L*) [12,13]. Recent partial characterization of *MM* of HbGp by matrix assisted laser

desorption time-of-flight mass spectrometry (MALDI-TOF-MS) confirmed the similarity of its subunits to those of homologous proteins of this class, mentioned above, especially HbLt [15]. This characteristic multi-subunit content confers to the whole protein a double-layered hexagonal oligomeric structure. A common model for the quaternary structure, so-called "bracelet model", has been employed to explain the assembly of this class of proteins into their oligomeric structure.

It is worth of notice that HbGp belongs to the same class of hemoglobins as HbLt, which is one of the most studied hemoglobins in this group. Despite the fact that HbLt has been extensively studied over the past 20 years, the issue of its true *MM* is still not fully understood. HbLt was one of the proteins studied by Theodore Svedberg and collaborators in 1933 [16], and later work by Daniel et al. has argued that the *MM* of HbLt could vary between 3.6 and 4.4 MDa [17]. They propose a model for the whole protein, consisting of twelve equal structures involving a dodecamer, $(abcd)_3$, and three linkers L_3, together with twelve tetramers $(abcd)$, in such a way that the protomer corresponding to the 1/12 of the whole oligomer is given by $(abcd)_3L_3(abcd)$, or alternatively, $(abcd)_4L_3$ [17,18]. On the other hand, Vinogradov et al. [19] have proposed a model for HbLt, where 1/12 of the whole molecule is given by $(abcd)_3 L_3$, so that the difference between the two models is the presence of twelve additional tetramers in the former occupying the central part of the hexagonal bilayer.

Royer et al. describing the crystal structure of HbLt [11,20] has suggested that the Vinogradov model is very consistent with the crystal structure. Besides, very recent preliminary work on the crystal structure of HbGp [21] has also suggested a strong similarity between HbGp and HbLt, both belonging to class 1, where the two hexagonal layers forming the bilayer are rotated to 16 degree one relative to another. It is worthy of mention, that another extracellular hemoglobin from a sea worm, HbAm, has also been investigated by Zal et al. [22,23]. In this case the structure belongs to class 2, where the two hexagonal layers are exactly eclipsed one relative to the other, as demonstrated in recent crystallographic studies at low resolution [24]. Besides, the assembly of the protomer corresponding to 1/12 of the whole HbAm oligomer is different: a dodecamer formed by two monomeric units a_1 and a_2 together with a trimer (T) gives $(a_1)_3(a_2)_6T$ associated to an average of 3.5 linkers resulting in a structure of $[(a_1)_3(a_2)_6T]L_{3.5}$ [23,25]. This arrangement implies a different number of globin and linker chains for HbAm. In this assembly the contacts between the subunits are quite different as compared to those in HbLt. Moreover, quite recently, the primary sequence of a fourth linker chain for HbLt has been reported, L_4 [1]. The unanswered question remains as an open issue regarding the role of four linker chains in a structure that, apparently, only needs three of them. Another interesting question, relevant to the understanding of the overall oligomeric subunit stoichiometry, is the existence of several isoforms for some of the globin chains. This has also been elusive in the description of the crystal structure of the whole assembly at atomic resolution. So, the precise characterization of the several globin and linker subunits that constitute the native extracellular hemoglobins still remains a matter for further research.

In conclusion, this review chapter addresses the issue of the molecular masses of HbGp subunits, as monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and MALDI-TOF-MS, and their hydrodynamic properties, as monitored by analytical ultracentrifugation (AUC). Also new data from other three giant hemoglobins are

added, to compare the differences and similarities between these systems: namely hemoglobins of *Rhinodrilus alatus*, *Eisenia andrei* and *Perionyx excavatus*. The focus of this study is the characterization of the different subunits that constitute each protein, aiming to assess in detail the nature of the subunit interactions that maintain the whole extraordinary oligomeric structure. These three new hemoglobins belong to different worm species that have been classified and studied previously only in relation to their biological effects upon the soil condition in their living habitat. Comparing the composition and structures of their subunits could be interesting also in relation to the understanding of the evolutionary changes evolved as a function of their different living environments. Finally, recent results concerning the subunits composition and architecture of HbAm, in connection with its possible medical application as a blood substitute, are described.

1.1 Some new extracellular hemoglobins from different worms

The giant extracellular hemoglobins found in the Annelids, are characterized by high molecular mass and by an overall hexagonal symmetry [23]. Hemoglobin provides a fascinating example of molecular evolution. In this work four types of extracellular hemoglobins from different earthworms were studied.

These species of worms have very specific habitat and are found and live only in some restricted regions. It is not possible to produce some of these earthworm species in large amounts and captivity. *Glossoscolex paulistus* is a worm of the Glossoscolecidae family, very common and endemic in the cities of Piracicaba and Rio Claro, in state of São Paulo, Brazil [26]. The more common use of these earthworms is in the fishing, as baits. However, the extracellular hemoglobin of *Glossoscolex paulistus*, HbGp, has now been studied for more than twenty years, focusing its physical-chemical and structural properties, by several research groups in Brazil [27,28].

Rhinodrilus alatus is a species endemic and prevalent in sites in the regions of Sete Lagoas and Paraopeba, in the state of Minas Gerais, Brazil [29]. This species was considered to be threatened of extinction, due to its extensive use to achieve natural fertilizer as well as for bait in fishing. This species was well characterized and its anatomy is known [30], especially regarding the circulatory system. *Rhinodrilus alatus* is a giant earthworm, of 56 to 63 cm of length and 11 to 12 mm of diameter [30]. In the period of drought, between April and September, the earthworms of this species enter into spontaneous dormancy, in a camera protected by mucus. *Rhinodrilus alatus* belongs to the same family as the *Glossoscolex paulistus* worm. Differently from HbGp, the extracellular hemoglobin of *Rhinodrilus alatus*, HbRa, is not known from the structural point of view.

Perionyx excavatus is a worm of the family Megascolecidae and genus Perionyx. *Perionyx excavatus* is an earthworm found commonly over a large area of tropical Asia although it has been transported to Europe and North America [31]. This is an endemic species which lives in organic wastes and high moisture contents, and adequate amounts of suitable organic material are required for populations to become fully established and for them to process organic wastes efficiently [32].

Perionyx excavatus had been shown to have efficient biological potential for conversion of organic wastes into high-value useful plant growth media [32]. Differently from the other two species, the *Perionyx excavatus* is produced in earthworm culture and its marketing is

allowed around the world. For this reason, it is often used in the process of vermicomposting, which is a mesophilic process associated to ingestion, digestion, and absorption of organic waste carried out by earthworms followed by excretion of castings through the worm's metabolic system [32].

Another extracellular hemoglobin that is discussed in this work is collected from the annelid *Eisenia andrei*, HbEa. This is an earthworm of the family Lumbricidae and is known as the red California worm. This earthworm is between 5 to 10 cm in length and 4 to 8 mm in diameter, and it is the most used species in the world for decomposition of organic material [33]. The easy adaptation and reproduction of this species partly justify the *Eisenia andrei* large use around the world.

Some interesting studies suggest that earthworms can be used as biological indicators of soil contamination. Vampré et al. [34] showed that soil contamination by hexachlorobenzene (HCB) can be identified by analysis of soil and earthworm tissue extracts [34]. The earthworm has an important role in the equilibrium of the soil nutrients distribution, in particular, the increase of soil stability, infiltration rates, besides playing an important role in the phosphorus (P) and nitrogen (N) cycles [35]. In this context, interesting work has been developed by Brown et al. regarding the worms population of the state of Paraná, Brazil, aiming to evaluate the soil characteristics of this region [36].

1.2 Extracellular worm hemoglobins as potential blood substitutes

Giant extracellular hemoglobins are easily purified to a homogeneous product avoiding expensive synthetic steps. Besides, it is a product easy to store, the auto-oxidation is quite a slow process and it is less likely to cause immunogenic responses since cell membranes are not present and HbAm, for instance, is not glycosilated [27]. Preliminary experiments have been performed many years ago with the naturally polymerized HbLt. Mice and rats undergoing exchange transfusion with HbLt revealed no apparent behavioral and physical changes [9]. A report on the potential use of HbAm, showing that its structural and functional properties are consistent with the requirements for blood substitutes, has been published recently [6]. These properties should prevent kidney damage, reduce vasoconstrictor effect and oxygen radical formation, often associated with the administration of dissolved mammalian Hb.

Structural analyses of HbAm under simulated human physiological conditions *in vitro* revealed its dissociation into high molecular weight and functional subunits, while *in vivo* experiments performed on mice revealed no apparent behavioral or physiopathological changes and absence of allergic response [6]. Recent effort has also been devoted to express some of the globin chains of HbAm considering the possibility of producing larger amounts of material aiming at reconstituting a recombinant hexagonal bilayer hemoglobin (HBL-Hb) by genetic engineering in order to provide a new oxygen carrier for therapeutic applications [10]. Considering the similarity of HbGp and HbLt with HbAm it is possible to conclude that all of them could be potentially useful for blood substitutes. Therefore, our studies aiming to obtain a deeper understanding of the structural and physical-chemical properties of HbGp and the new giant hemoglobins presented in this report can be quite relevant to a better evaluation of their potential biomedical applications.

2. Experimental methods

In the present work, a review is presented of recent work performed in our laboratory, focusing on the molecular mass MM of HbGp, based on electrophoresis, AUC, and MALDI-TOF-MS [3,15]. SDS-PAGE electrophoresis is an extremely useful technique, especially to monitor the subunits contents present in different fractions of the protein obtained from gel exclusion chromatography [26]. Though this technique is not as precise as direct mass spectrometry, it is able to provide informations about the mass characteristic and migration properties of these subunits under the effect of an applied electric field at the same time.

X-Ray crystallography is a powerful structural technique, providing models of complex protein structures with atomic resolution [21].

3. Results

3.1 Equilibrium of HbGp species obtained from alkaline dissociation at pH 10

Our recent MM determination performed on two HbGp samples, at pH 7.0, one in the reduced oxy- form, and the other in the oxidized cyanomet- form, have given values of 3.6 ± 0.1 MDa and 3.7 ± 0.1 MDa, respectively [3]. This value is in excellent agreement with the prediction of the MM for the whole molecule, based on the Vinogradov model, and our previous MALDI-TOF-MS analysis [15]: a tetramer *abcd* of 52.1+16.4 = 68.5 kDa is observed, and assuming further an average MM for the linkers of 28 kDa, plus 144 heme groups (~0.6 kDa each), a total MM of 12×[68.5×3+28×3]+144×0.6 = 3560 kDa is expected for HbGp.

Very recent ultracentrifugation studies regarding the HbGp subunits, obtained from alkaline dissociation at pH 10, provided further information on subunits masses, underlining the difficulties in separating, by simple SEC experiments, the linker chains from the trimer globin subunits due to their similar hydrodynamic properties [37]. In this study, analysis of the equilibrium dissociation of HbGp, at pH 10.0, has shown that the whole protein is constituted by several species. For oxy-HbGp, at pH 10.0, no contribution from un-dissociated whole protein is observed, suggesting that the reduced form is completely dissociated under this alkaline condition. However, for cyanomet-HbGp, 17 % of the protein remains in the native un-dissociated form, implying a higher oligomeric stability of the oxidized cyanomet- form [37]. Results are shown in Tables 1 and 2 and can be summarized as follows: 1) AUC experiments for the monomer *d* alone indicated that this species is quite pure. However, equilibrium is observed involving monomers and dimers of monomers. The dimer contribution is relatively low, around 10%, similar to that observed in previous experiments by MALDI–TOF-MS, increasing at higher protein concentrations. 2) Analysis of oxy-HbGp solution, at pH 10.0, showed the existence of four species in equilibrium, assigned to the following subunits: monomer *d*, dimers of monomers d_2, trimers *abc* and a fourth species associated to the tetramer *abcd*. The second species (dimers of monomers, d_2) revealed a significant intensity increase as compared to the pure monomer. This increase in intensity is, possibly, due to a superposition of the contribution from some linker chains, having masses near to the dimers of monomers d_2 one, around 32 kDa. Linker chains are characterized by mass values MM in the range of 24-32 kDa, and are, probably, detected as a single peak, corresponding to the peak of the dimers of monomers. The third species, trimers *abc*, is not observed for samples containing the reducing agent, β-mercaptoethanol,

since the disulfide bonds are disrupted with the formation of the monomeric species that compose the trimers, monomers *a, b* and *c*, consistent also with the increase in the contribution of the monomeric fraction, observed for this sample (Tables 1 and 2). Calculations of the sedimentation coefficients for the HbLt subunits based on the reported crystal structure [11,20] gave additional useful informations for the interpretation of the obtained AUC data for HbGp: first of all, according to these estimates, the linker chains could appear together either with the monomeric globin chains or with the dimeric globins. Besides, the presence of linker chains associated to globin tetramers would not alter in significant ways the value of s for the pure globin tetramer.

Samples	pH	Observed species[a]				
		$s^0_{20,w}$				
		1	**2**	**3**	**4**	**5**
Monomer	7.0	2.05 ± 0.05	2.81± 0.03	-	5.7 ± 0.1	
	10.0	1.86 ±0.06	2.9 ± 0.5	-	4.6 ± 0.9	
Oxy-HbGp	10.0	1.7 ± 0.1	2.67 ± 0.03	4.12±0.06	6.2 ± 0.2	
Oxy-HbGp[1]	10.0	1.64 ± 0.06	2.67 ± 0.02	-	4.6 ± 0.2	
Cyanomet-HbGp	10.0	2.0 ± 0.1	3.06 ± 0.09	3.98 ±0.02	6.6 ± 0.2	58.3 ± 0.5

Oxy-HbGp[1] –HbGp in the presence of 2-mercaptoethanol. All data obtained at 236 nm. [a]Observed species 1, 2, 3, 4 and 5 correspond to pure monomer *d*, dimer of monomers d_2, trimer *(abc)*, tetramer *(abcd)* and tetramer of monomers d_4 and un-dissociated cyanomet-HbGp, respectively.

Table 1. Sedimentation coefficients for the pure monomer **d**, and for HbGp, dissociated in alkaline medium, under the indicated conditions.

Samples	pH	Observed species[a]				
		% (Area)				
		1	**2**	**3**	**4**	**5**
Monomer	7.0	84 ± 8	14 ± 8	-	2 ± 1	-
	10.0	89 ± 4	8 ± 4	-	3 ± 1	-
Oxy-HbGp	10.0	24 ± 8	29 ± 10	38 ± 3	9 ± 6	-
Oxy-HbGp[1]	10.0	47 ± 4	38 ± 3	-	15 ± 3	-
Cyanomet-HbGp	10.0	20 ± 4	20 ± 7	40 ± 3	3 ± 1	17 ± 2

Oxy-HbGp[1] – HbGp in the presence of 2-mercaptoethanol. All data obtained at 236 nm. [a]The species are the same described in Table 1.

Table 2. Percentage contributions of different species observed in the equilibrium of HbGp, dissociated at alkaline medium, pH 10.0, and of monomer *d*, shown in Table 1.

As mentioned above, for oxy-HbGp no contribution from un-dissociated protein was observed, implying that the reduced form is fully dissociated at pH 10.0. However, for cyanomet-HbGp, around 17 % of un-dissociated protein remains in its native oligomeric form, consistent with a greater oligomeric stability for this oxidized form, in alkaline medium (Tables 1 and 2). Finally, equilibrium sedimentation data allowed an estimate of the masses *MM* for the four globin chains, monomers *a*, *b*, *c* and *d*. Due to the small difference of masses for monomers *b* and *d* they appear as a single unresolved peak in AUC and MALDI-TOF-MS [15] experiments.

3.2 Initial characterization of the new extracellular hemoglobins from different worms

In this section new data are presented regarding both electrophoretic and MALDI-TOF-MS measurements of HbGp subunits. Four linker chains are observed for this species by SDS-PAGE, but with quite different concentrations that preclude their detection in the mass spectra experiments. Moreover, multiple chains were detected for the monomeric *c* chain obtained by reduction of the trimer *abc*, as for the isoforms of monomeric subunit *d*, reported earlier [15]. Together with the studies on HbGp, we have recently started to look at three different hemoglobins obtained from different worms, namely HbRa, HbPe, and HbEa. Some preliminary results based on SDS-PAGE, AUC, and MALDI-TOF-MS studies are here reported for the first time, showing a great similarity, but not identity, among the subunit composition and masses for these extracellular hemoglobins as compared to HbGp.

3.2.1 SDS-PAGE electrophoresis data

In Figs.1A and 1B SDS-PAGE gels are shown for the extracellular hemoglobins of HbGp, HbRa, HbPe and HbEa, in the absence and the presence of 2-mercaptoethanol, respectively. The slot (S) corresponds to the standard masses used for calibration. The four hemoglobins in the present study showed a similar, but not identical, band pattern with mass values around 52 ± 2 kDa, associated to the trimer subunit, and also very similar masses for the monomer *d*, around 12 kDa (Fig.1A). The slots II and III correspond to HbGp. The slots IV and V, corresponding to HbRa, showed some differences as compared to HbGp: apparently two bands were observed in the trimer position, while the four linker subunits with different intensities were shifted as compared to HbGp bands. Furthermore, two linkers showed masses similar to those of HbGp and the other two smaller ones. The HbPe bands, shown in slots VI and VII, presented three linker chains, one of them with a higher mass as compared to HbGp and HbRa (mass value around 37 ± 2 kDa). For the HbEa bands presented in slots from VIII to X, also three linker chains were observed, two of them with masses very close to those of HbRa and the third one with a greater one (around 49 kDa).

In Fig.1B a SDS-PAGE gel image is shown for the four different hemoglobins, in the presence of the 2-mercaptoethanol. The absence of the band corresponding to the trimer around 52 kDa (see Fig.1A) was observed, as a result of the disulfide bond reduction into the three corresponding monomeric subunits. The linker pattern for HbGp and HbRa were essentially maintained upon addition of the reducing agent (slots II-III and IV-V, respectively). In the slots IV and V it was noticed that the monomeric subunits *a*, *b* and *c* for HbRa were similar to those for HbGp. HbRa had two of these monomers with mass values very close, basically unresolved as a single broad band. HbPe, shown in slots VI and VII, presented one of the trimer monomeric subunits heavier than that for HbGp and HbRa.

However, the other two reduced monomeric subunits from the trimer were very similar, as observed for HbGp and HbRa. The linkers pattern for HbPe changed upon disulfide bonds reduction in the presence of the 2-mercaptoethanol. It is not clear if this was only due to partial denaturation of the protein in the presence of the reducing agent, leading to a change in protein migration.
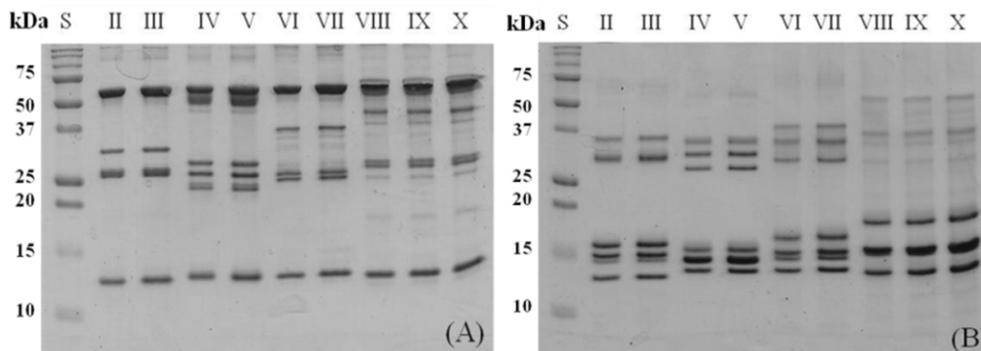

Fig. 1. SDS-PAGE gel electrophoresis for the different extracellular hemoglobins analyzed in this work. The gel concentration was 15% in 25 mmol/L Tris-HCl, 192 mmol/L glycine, pH 8.3, and stained with Comassie Blue R-250. The slot (S) corresponds to the standard masses. (A) Gel electrophoresis in the absence of 2-mercaptoethanol and (B) with the reducing agent. The slots II and III correspond to HbGp, slots IV and V to HbRa, VI and VII to HbPe, and VIII, IX and X to HbEa.

Based on SDS-PAGE, HbEa is the hemoglobin that presents subunits with the highest mass values. All the monomeric subunits obtained from the trimer are heavier as compared to the other hemoglobins studied in this work. Its monomeric subunits have mass values in the range from 12.5 to 18.5 kDa (Fig. 1B). It is also observed one of the linkers with MM of 47 ± 2 kDa. These heavier subunits should confer a higher value of MM to the whole HbEa as compared to HbGp, HbRa and HbPe. Although, some heavier chains are observed in HbPe and HbEa, it seems that no stoichiometry change occurs for the whole oligomer as compared to HbRa, since the same number of subunits is observed when the disulfide bonds are reduced: namely, four globin monomers and four linkers are detected. This implies that the hemoglobins HbRa, HbPe and HbEa should have a stoichiometry for the whole protein of $[(abcd)_3L_3]_{12}$. Nevertheless, for HbGp only two linker chains are observed in the SDS-PAGE, but, probably, each observed band contains two linkers superposed or, alternatively, the amounts of two of the linkers are too low to be observed in the gel.

Interestingly, some differences observed in the migration for the monomeric subunits d for all the studied hemoglobins, in the presence of the reducing agent 2-mercaptoethanol, can be associated to the higher unfolding of these subunits, due to the reduction of all disulfide bonds in the hemoglobins. For this reason, when the subunits undergo partial unfolding, their migration through the SDS-PAGE gel becomes more difficult. Being SDS-PAGE electrophoresis alone rather limited, more detailed characterization of the subunits for these hemoglobins by mass spectrometry was performed.

3.2.2 MALDI-TOF-MS data

Fig.2 shows the MALDI-TOF-MS spectrum of a solution of hemoglobin of *Rhinodrilus alatus*, at pH 7.0, using sinapinic acid as matrix in positive ion mode. Fig. 2A shows the peaks obtained for the monomer d subunit, the trimer (abc) and the linker chains. In agreement with previous studies of hemoglobin of *Glossoscolex paulistus* [15], Fig. 2A shows an intense peak centered around 16.3 kDa, corresponding to the monomer d, which is consistent with its relatively easy ionization. Fig.2B shows the expanded region from 15,500 to 18,200 Da, corresponding to the single protonation of the monomer d. The contribution of two more intense isoforms, d_1 and d_2, and another less intense isoform d_3 is evident in Fig.2B.
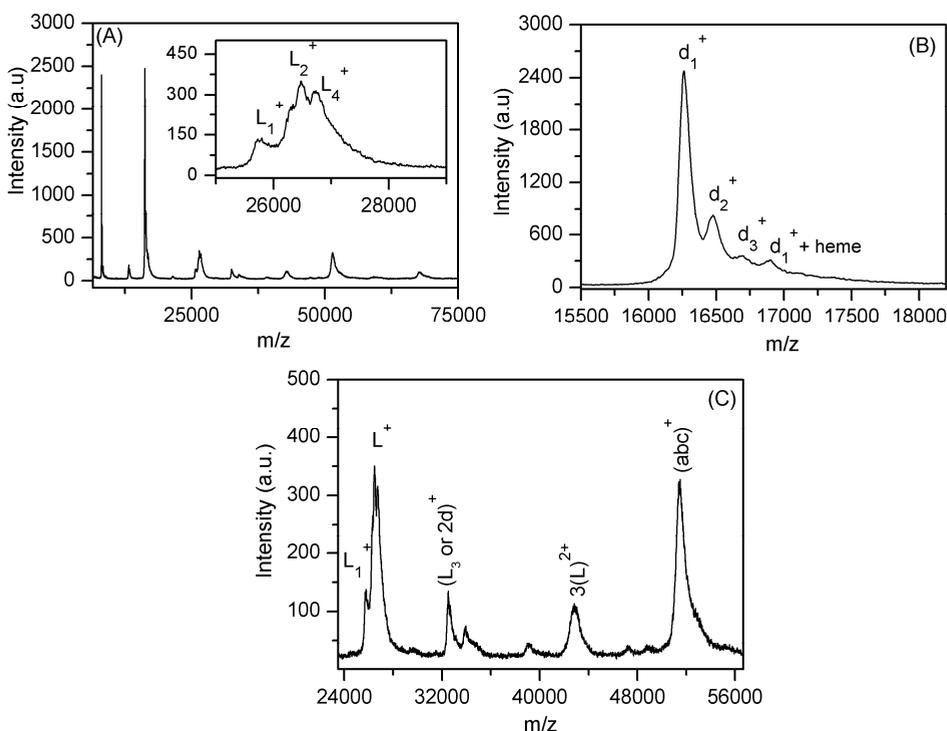
Fig. 2. (A) MALDI-TOF-MS spectrum of *Rhinodrilus alatus* hemoglobin, HbRa, at pH 7.0. The insert shows the expanded ordinate, corresponding to the intensity in arbitrary units, highlighting the linkers peaks; (B) the expanded region for the mono-protonated monomer d^+ from 15,500 to 18,300 Da; (C) the expanded region for the mono-protonated trimer abc^+ and linkers from 24,000 to 56,000 Da.

Moreover, a small contribution around 16,900 ± 30 Da can be associated to d_1 + heme, which is not observed for the other isoforms. In Fig. 2C the expanded region from 24,000 to 56,100 Da is displayed, corresponding to the single protonation of the linker chains L and trimer subunits (abc). The spectrum clearly shows the existence of three linker chains L_1, L_2 and L_4, with 25,770, 26,540 and 26,740 Da, respectively. The remaining linker chain, L_3, at 32,515 Da,

can be superposed with the dimers of monomer $2d$. Finally, an intense contribution at 51,470 Da is observed, associated to the trimer $(abc)^+$, while the less intense species at 67,690 Da is due to the contribution of the tetramer $(abcd)^+$ (Table 3).

Our results for HbRa suggest that the molecular masses of its subunits are very similar to those for HbGp, but presence of two isoforms for the trimer subunit, as noticed for HbGp [15], is not observed in this hemoglobin. This might indicate that the monomers a, b and c do not have isoforms.

In Table 3, the results of analysis of MALDI-TOF-MS data for hemoglobin of *Eisenia andrei* are shown. In Fig. 3A the peaks obtained for the mono- and di-protonated monomer d subunit are significantly more intense as compared to the trimer (abc) and the linker chains ones. The molecular mass of the monomer d subunit is very similar to those for the HbGp and HbRa hemoglobins, but the peak corresponding to d_1 bound to the heme is not observed. In Fig.3B the three linker chains L_1, L_2 and L_4, with masses of 25,110, 26,470 and 27,445 Da, respectively, and another linker L_3 with a more intense peak at 32,775 Da, are shown. Two additional contributions at 53,270 ± 80 and 69,590 ± 100 Da are also shown in Fig. 3C, which can be assigned to the trimer (abc) and tetramer $(abcd)$, respectively. Differently from the HbRa and HbGp, the hemoglobin of *Eisenia andrei* has only one predominant monomeric isoform d_1 with molecular mass at 16,344 ± 24 Da. The remaining monomeric isoforms have low intensities.

Mass of the extracellular hemoglobins in (Da)				
Subunits	HbGp	HbRa	HbEa	HbPe
d_1	16,370	16,272 ± 16	16,344 ± 24	16,220 ± 19
d_2	16,415 ± 10	16,490 ± 20	16,495 ± 12	16,438 ± 20
d_3	16,650 ± 40	16,710 ± 25	16,560	16,656 ± 25
d_4	16,850 ± 40	-	16,790	-
$d_1 + heme$		16,900 ± 30		16,844 ± 20
L_1	25,780 ± 30	25,770	25,110	25,670
L_2	26,750 ± 80	26,540	26,470	27,145
L_4	-	26,740	27,445	-
L_3 or $2d^*$	32,870	32,515	32,775	32,410
T_1 (abc)	51,200	51,470	53,270 ± 80	51,240
T_2 (abc)	51,985	-	-	-
abcd	68,400	67,690	69,590 ± 100	67,620

*The value of mass corresponds very closely to the dimer of the monomer isoform d_2

Table 3. Molecular masses in (Da) of extracellular hemoglobin subunits, obtained from MALDI-TOF-MS, and for different worms.
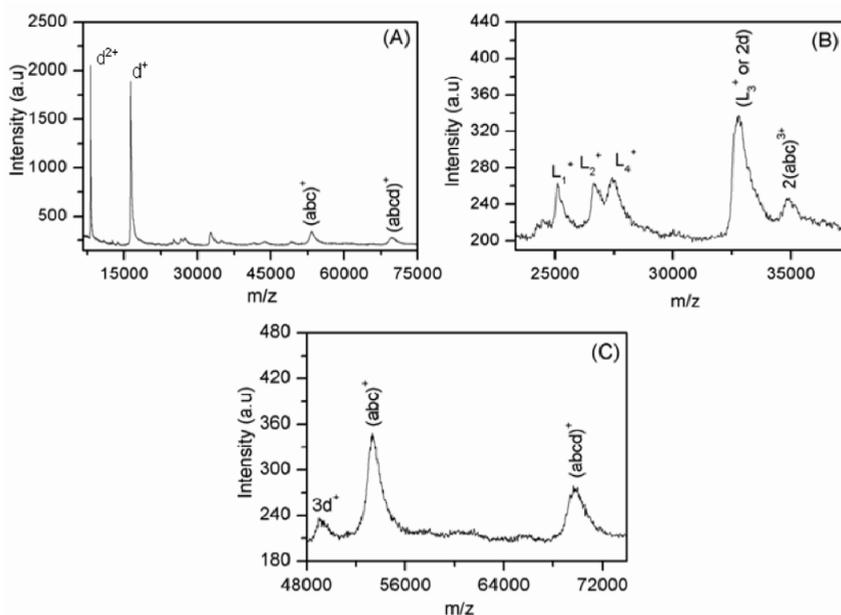
Fig. 3. MALDI-TOF-MS spectrum of *Eisenia andrei* hemoglobin, HbEa. (A) at pH 7.0, full mass range. (B) Corresponds to the intensity in arbitrary units, highlighting the linkers and triple-protonated dimer of trimer; (C) the expanded region for the mono-protonated trimer abc^+ and tetramer $abcd^+$ from 48,000 to 75,000 Da.

Our results indicate that the HbEa presents two significant differences when compared with the HbGp: one of the monomeric chains *a*, *b* or *c*, that constitute the trimer *abc*, has a molecular mass higher than the corresponding HbGp one, due to the mass difference of 2 kDa observed in the trimer of the HbEa (Table 3). The second is the presence of one additional linker chain (L_4) in the oligomeric structure of HbEa.

The data for hemoglobin of *Perionyx excavatus* are shown in Table 3. In this case the molecular mass of monomer *d* is analogous to that observed for the other hemoglobins. Moreover, it was noticed that a small proportion of the monomer *d* remained associated to heme with a mass of 16,844 ± 20 Da. In general, the subunits of the HbPe are very similar to those of HbGp, since no significant differences in the molecular masses are observed. Very recent studies of HbGp, in the presence of 2-mercaptoethanol [38], have shown that the monomer *c* chain presents also four isoforms with *MM* in the range of 17.3-17.6 kDa. This is, probably, why the trimer **T** is also characterized by two isoforms (Table 3). Further experiments in the future, in the presence of 2-mercaptoethanol will be necessary to elucidate more clearly the masses differences of the globin subunits forming the trimer *abc*, especially regarding the new hemoglobins.

3.2.3 AUC data

All AUC experiments were performed in 100 mmol/L Tris-HCl containing 50 mmol/L NaCl, at pH 7.0 or 5.0, and at 20 ºC. The protein concentrations were in the range 100 to 300

μg/mL, and the speed rotor was 20,000 rpm. The SEDFIT software was used in the analysis of sedimentation velocity (SV) data. The V_{bar} parameter used in the fits was the standard value of 0.733 mL/g and f/f_0 was allowed to float being a regularization parameter.

Fig.4 shows sedimentation coefficient distributions c(S), at 200 μg/mL, for the four extracellular hemoglobins. Our results indicate that three of the four hemoglobins present a good homogeneity with a single species in solution. However, for the extracellular hemoglobin of *Eisenia andrei* two different populations are observed, the first one around 10 S can be associated to the dodecamer subunits while the other species at 64 S corresponds to the whole protein. Although these proteins are quite stable in these conditions [3], the observed dissociation is, probably, due to the purification process used for this hemoglobin.
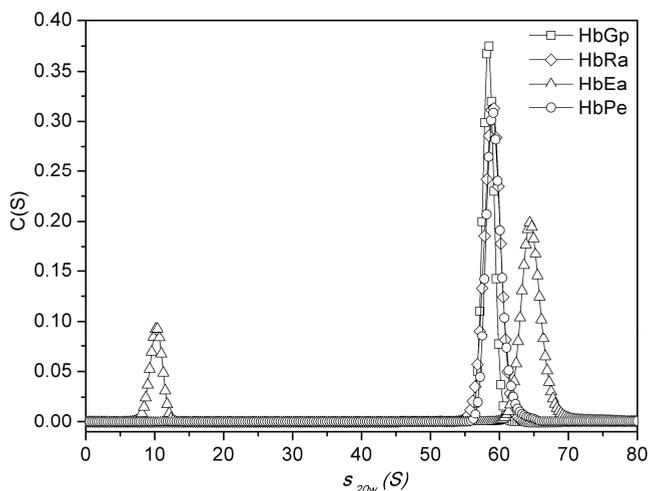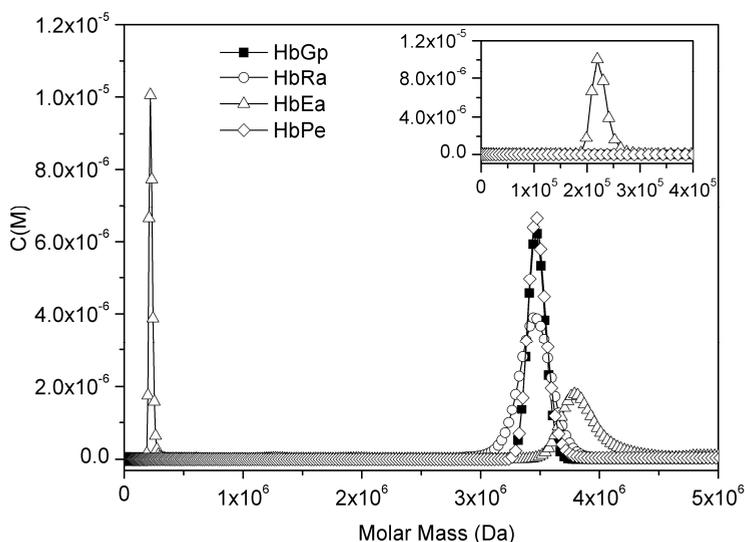

Fig. 4. Continuous sedimentation coefficient distribution of extracellular hemoglobins. The c(S) fittings for hemoglobins, at a concentration of 200 μg/mL, in 100 mmol/L Tris–HCl containing 50 mmol/L NaCl, are shown. The $s_{,20w}$ for each fitted curve was determined as the maximum of the peaks of c(S) curves. Absorbance was monitored at 415 nm.

The work of Krebs et al [39] on the properties of the dodecamer subunit, $(abcd)_3$, of HbLt showed that the dodecamer obtained from urea dissociation of the whole protein consisted of an equilibrium of three species with sedimentation coefficients of 8.5 - 9.4 S, 3.6 - 4.4 S and 1.9 S. According to our AUC data for HbGp, the dodecamer is associated to the 8.5 - 9.4 S species. The other two species correspond to trimers and monomers, in order of decreasing s, implying that urea dissociation in HbLt [39] produces an equilibrium of three species.

The s values were corrected to standard conditions (water and 20 ºC), and the $s^0_{20,w}$ values, corresponding to $s_{20,w}$ at 0 mg/mL protein, were obtained by linear regression extrapolation. The $s^0_{20,w}$, values for the oxy-HbGp at pH 5.0 and 7.0 were 58.6 ± 0.4 and 58.1 ± 0.4 S, respectively, as shown in Table 4. These results suggest that HbGp is very stable in both pH values, and does not undergo oligomeric dissociation at pH 5.0. For the other two hemoglobins, HbRa (*Rhinodrilus alatus*) and HbPe (*Perionyx excavatus*), similar results were observed as for HbGp [3], with $s^0_{20,w}$ around of 59 S. The mass values are very similar, within the experimental error, with the exception of HbEa that has a higher mass (Table 4).

Hemoglobins	$s_{20,w}^{0}(S)$	*MM* by c(*M*) **(kDa)**
Glossoscolex paulistus (HbGp)[1]	58.6 ± 0.4	3600 ± 80
Glossoscolex paulistus (HbGp)[2]	58.1 ± 0.4	3500 ± 100
Rhinodrilus alatus (HbRa)	59.1 ± 0.1	3500 ± 70
Eisenia andrei (HbEa)	64.6 ± 0.4	3850 ± 50
Perionyx excavatus (HbPe)	58.9 ± 0.2	3500 ± 50

[1]HbGp at pH 5.0. [2]HbGp at pH 7.0.

Table 4. AUC hydrodynamic data analysis, for extracellular hemoglobins from different worms, at 20.0 ± 0.1 °C and pH 7.0.

In Fig.5 the c(M) distributions are shown for the four types of studied extracellular hemoglobins, at a protein concentration of 300 μg/mL. It is noticed that HbEa has a higher *MM* than the other three hemoglobins one (Table 4). This 330 kDa difference can be attributed to two factors: 1) the difference of the subunits masses constituting the oligomeric structure, and 2) a different subunit stoichiometry for the HbEa oligomer. Thus, the inclusion of another linker chain in the structure of HbEa, giving a model like $[(abcd)_3 L_4]_{12}$, could contribute with a 326 kDa increase in the total mass of the molecule, consistent with the results observed for HbEa in the c(M) values. This fourth linker chain would have an average mass of 326/12 =27 kDa.

Fig. 5. Continuous molecular mass distribution of extracellular hemoglobins. The c(M) fittings for hemoglobins, at a concentration of 300 μg/mL, in 100 mmol/L Tris–HCl containing 50 mmol/L NaCl are shown. The insert shows the enhanced plot in the *MM* range from 0 to 400 kDa. The *MM* for each fitted curve was determined as the maximum of the peaks of c(M) curves. Absorbance was monitored at 415 nm.

In the c(M) distribution curve for the hemoglobin of *Eisenia andrei* an additional species of 210 kDa is observed, which is assigned to the dodecamer (*abcd*)$_3$. This mass value is in agreement with the literature reports of 205 kDa [19]. Moreover, the peak at 3.8 MDa corresponding to the whole native protein is broadened, which is, probably, due to a superposition of two contributions: from species lacking the dodecamer and from the whole protein.

The *MM* of three of the hemoglobins shown in Table 4 is around 3.6 MDa, indicating that these proteins have the same stoichiometry and subunits with quite similar mass. Moreover, these *MM* values strongly suggest that the Vinogradov's model [(*abcd*)$_3L_3$]$_{12}$ [19] is adequate for the hemoglobins of *Glossoscolex paulistus*, HbGp, *Rhinodrilus alatus,* HbRa, and *Perionyx excavatus,* HbPe. However, our results indicate that for the hemoglobin of *Eisenia andrei* this model is not appropriate, since a significant mass difference is observed corresponding to 12 additional linker chains (with an average mass of 27 kDa).

3.3 Structural model and stoichiometry

The first crystallization experiments with giant hemoglobins are dated from 1840, when crystals from HbLt were obtained. Indeed, HbLt was the first protein crystallized ever [40]. Nevertheless, it took more than one hundred and sixty years to obtain a complete crystallographic structure [20]. The crystal structure of HbLt, at 3.5 Å of resolution, elucidated how the hierarchical levels of an erythrocruorin is made, and represented a fundamental step to better understand these structures. Recently, our group, reported the preliminary structure of HbGp, at 3.15 Å of resolution [21]. The HbGp and HbLt share an estimated identity for the monomer subunit *d* of 54 % [41], with the HbGp structure showing the same hierarchical levels as HbLt (Fig. 6), and the same global association, into type 1. It was the second time that an entire type 1 erythrocrurin has been reported. As in HbLt, the electron density map of the HbGp reveals important details, such as the conserved calcium biding sites, inter/intra chains disulfide bonds and heme group positions. Very recently, crystals from the giant hemoglobins from HbEa, and HbPe were also obtained in our group and the preliminary structure for HbEa has been solved, with a resolution of 4.7 Å, which is enough to conclude that, like HbGp and HbLt, HbEa also belongs to type 1 array. Further experiments are needed to improve the resolution and find evidences on the electron density map where HbEa shows an accumulation of mass [42].

Another very recent relevant advance in the structural analysis was the characterization of the isolated subunit *d* from HbGp. In the biological unit, subunits type *d* form an interface with subunits type *a*, involving the heme groups from both chains, and at the same time an interface with two other subunits *d*, forming a trimeric array, as shown in Fig. 6. We showed that once isolated, the subunit *d* shows, as a first level of aggregation, a dimer *d-d*, with an interface involving heme groups, similar to the one observed between *a-d* in the biological unit (where *a* and *d* shares 27% of identity). In the next level, the crystal lattice shows a stable dimer of dimers *d-d. T*his tetrameric array is unrelated with the hetero tetramer (*abcd*) in the biological unit [43]. The existence of these oligomeric structures has also been confirmed by AUC, which shows that the pure monomer *d* solution contains also three species, monomer, dimer and tetramer, and increasing the protein concentration the contribution of the monomeric species reduces from 90 to 80 % [44]. On the other hand, the trimer formed by the subunits *d* in the biological unit has not been observed for the isolated

subunit *d*, which leads us to conclude that such an arrangement is a consequence of associations between subunits *d* and *a* in the whole oligomer.
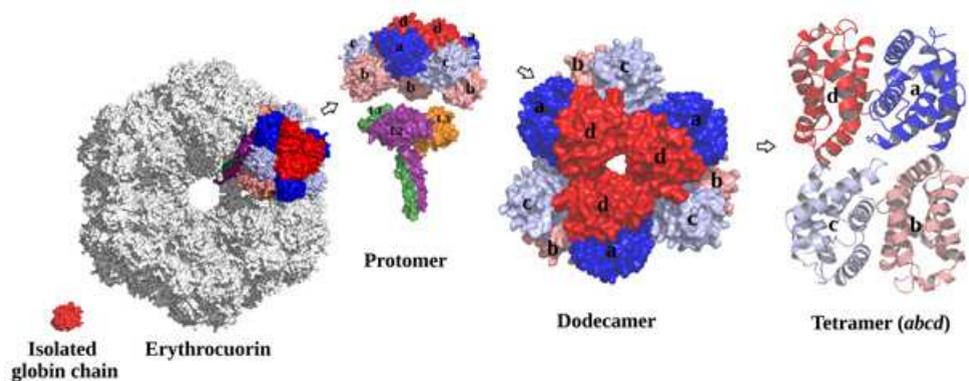

Fig. 6. Schematic representation of the hierarchy in the oligomeric assembly of HbGp into the native structure as obtained from crystallographic data analysis. The whole HbGp structure is composed by twelve protomers, each of them containing a dodecameric structure of globins and three linker subunits, $(abcd)_3L_3$. The dodecameric structure is composed by four types of globin chains: *a*, *b*, *c* and *d*. The four globin chains are associated as a hetero-tetramer (where *a*, *b*, and *c* form a trimer, linked by disulfide bonds).

4. Conclusions

In order to evaluate the potential usefulness of such a giant protein such as HbGp for biomedical applications, a detailed knowledge of its constituent subunits is very relevant. In the present review chapter, recent results for HbGp were described, based on the use of SDS-PAGE, MALDI-TOF-MS and AUC. Preliminary characterization of three new extracellular hemoglobins of different worm species was also described for the first time. The electrophoresis analysis of these hemoglobins shows that they have a subunits structure very similar to that described for HbGp. Moreover, our present results based on AUC data show that two of the new hemoglobins have a total *MM* similar to that of HbGp, namely 3.6 MDa, while the fourth one has a higher *MM* of 3.8 MDa. It appears clear from our data that the globin chains are a total of four with a trimer of 52 - 53 kDa and a monomer of 16 kDa as a common feature for all these hemoglobins. The highest *MM* hemoglobin, HbEa, has heavier linker chains as well as one of the globin monomers. MALDI-TOF-MS analysis for HbGp showed that the monomer *c* presents four isoforms and that the trimer, *abc*, is characterized by two isoforms, T_1 and T_2. In the final part of this review some crystal structures are also reported, which might be helpful in understanding the oligomeric structure of this class of proteins. Our present studies for HbGp are consistent with literature reports on several other extracellular hemoglobins, such as the HbLt and HbAm. Future more detailed characterization of the new hemoglobins will be important to assess all the subunit masses to model their oligomeric structure. We believe our results represent a nice contribution and an important and necessary step in the complete characterization of HbGp oligomeric structure.

5. Acknowledgments

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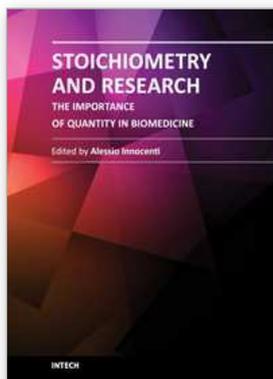
Stoichiometry and Research - The Importance of Quantity in Biomedicine

Edited by Dr Alessio Innocenti

The aim of this book is to provide an overview of the importance of stoichiometry in the biomedical field. It proposes a collection of selected research articles and reviews which provide up-to-date information related to stoichiometry at various levels. The first section deals with host-guest chemistry, focusing on selected calixarenes, cyclodextrins and crown ethers derivatives. In the second and third sections the book presents some issues concerning stoichiometry of metal complexes and lipids and polymers architecture. The fourth section aims to clarify the role of stoichiometry in the determination of protein interactions, while in the fifth section some selected experimental techniques applied to specific systems are introduced. The last section of the book is an attempt at showing some interesting connections between biomedicine and the environment, introducing the concept of biological stoichiometry. On this basis, the present volume would definitely be an ideal source of scientific information to researchers and scientists involved in biomedicine, biochemistry and other areas involving stoichiometry evaluation.

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