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On the molecular mass of the extracellular hemoglobin of *Glossoscolex paulistus*: Analytical ultracentrifugation reexamination

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ABSTRACT

The giant extracellular hemoglobin of *Glossoscolex paulistus* (HbGp) is constituted by subunits containing heme groups with molecular masses (*M*) in the range of 15 to 19 kDa, monomers of 16 kDa (*d*), and trimers of 51 to 52 kDa (*abc*) linked by nonheme structures named linkers of 24 to 32 kDa (*L*). HbGp is homologous to *Lumbricus terrestris* hemoglobin (HbLt). Several reports propose *M* of HbLt in the range of 3.6 to 4.4 MDa. Based on subunits *M* determined by mass spectrometry and assuming HbGp stoichiometry of $12(abcd)_3L_3$ (Vinogradov model) plus 144 heme groups, a value of *M* for HbGp oligomer of 3560 kDa can be predicted. This value is nearly 500 kDa higher than the unique HbGp *M* value reported in the literature. In the current work, sedimentation velocity analytical ultracentrifugation (AUC) experiments were performed to obtain *M* for HbGp in oxy and cyano-met forms. $s^0_{20,w}$ values of 58.1 ± 0.2 S and 59.6 ± 0.2 S, respectively, for the two oxidation forms were obtained. The ratio between sedimentation and diffusion coefficients supplied values for *M* of approximately 3600 ± 100 and 3700 ± 100 kDa for oxy and cyano-met HbGp forms, respectively. An independent determination of the partial specific volume, V_{bar}, for HbGp was performed based on density measurements, providing a value of 0.764 ± 0.008, in excellent agreement with the estimates from SEDFIT software. Our results show total consistency between *M* obtained by AUC and recent partial characterization by mass spectrometry. Therefore, HbGp possesses *M* very close to that of HbLt, suggesting an oligomeric assembly in agreement with the Vinogradov model.

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

The giant extracellular hemoglobin of *Glossoscolex paulistus* (HbGp) was characterized 20 years ago by ultracentrifugation with a minimum *M* of 3.1×10^6 Da and a sedimentation coefficient of 58 S [7]. This unique *M* estimate for HbGp has been an intriguing matter for a long time, requiring further studies and refinement, because from the beginning the reason for such a low value as compared with the *M* of similar homologous proteins was not clear [1–6]. These extracellular hemoglobins are constituted by a large number of subunits containing heme groups with *M* values in the range 15 to 19 kDa. These heme-containing subunits form monomers of 16 kDa (*d*) and disulfide-bound heterotrimers of 51 to 52 kDa (*abc*), linked by nonheme structures (24–32 kDa) named linkers (*L*) [3,5,8]. Recent partial characterization of *M* of HbGp by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) confirmed the similarity of its subunits to those of homologous proteins of this class mentioned above [9]. This characteristic multisubunit content confers to the whole protein a double-layered hexagonal oligomeric structure [8,10]. A common model for the quaternary structure, the so-called "bracelet model," has been employed to explain the assembly of this class of proteins into their oligomeric structure [11]. It is noteworthy that HbGp belongs to the same class of hemoglobins as *L. terrestris* (HbLt), which is one of the most studied hemoglobins in this group [11–18]. Despite the fact that HbLt has been studied extensively over the past 20 years, the issue of its true *M* is still under consideration. HbLt was one of the proteins studied by Svedberg and Eriksson [19], and recent work by Daniel and coworkers [16,20,21] argued that the *M* of HbLt could vary between 3.6 and

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[1] *Abbreviations used:* SOD, superoxide dismutase; HbLt, hemoglobin of *Lumbricus terrestris;* HbAm, hemoglobin of *Arenicola marina;* HbGp, hemoglobin of *Glossoscolex paulistus;* MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; HBL, hexagonal bilayer; AUC, analytical ultracentrifugation; SV, sedimentation velocity; DLS, dynamic light scattering; SAXS, small-angle X-ray scattering.

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4.4 MDa. They proposed a model for the whole protein, consisting of 12 equal structures involving a dodecamer $(abcd)_3$ and three linkers L_3, together with 12 tetramers (*abcd*), in such a way that 1/12 of the whole oligomer is given by $(abcd)_3L_3(abcd)$ or, alternatively, $(abcd)_4L_3$. The difference between the Daniel model and the Vinogradov model [12] is the presence of 12 additional tetramers in the former occupying the central part of the hexagonal bilayer (HBL).

Due to their extracellular nature, large size, and resistance to oxidation, erythrocruorins have been proposed as useful model systems for developing therapeutic extracellular blood substitutes, and preliminary animal experiments have been encouraging [4]. Studies focused on the giant extracellular hemoglobin of *A. marina* have shown excellent possibilities of application of this protein as artificial blood. This hemoglobin is easily available and can be purified to a homogeneous product, avoiding costly synthetic steps. The HBL hemoglobins are easy to store, their side effects are less pronounced, and they are less likely to cause immunogenic responses because cell membranes are not present in the preparations and the protein is not glycosylated. Furthermore, recombinant DNA techniques can be used to express the hemoglobin in large quantities [4].

In the current work, a reexamination of the total *M* of HbGp was performed using analytical ultracentrifugation. Based on our recent MALDI–TOF MS analysis [9], and considering that the Vinogradov model applies for HbGp, a tetramer *abcd* of 52.1 + 16.4 = 68.5 kDa is observed, and assuming further an average *M* for the linkers of 28 kDa plus 144 heme groups (~0.6 kDa), a total *M* of $12 \times [68.5 \times 3 + 28 \times 3] + 144 \times 0.6 = 3560$ kDa is expected for HbGp. It is important to notice that previous inconsistencies regarding the total *M* of HbLt were explained in the literature based on the fact that iron heme oxidation of the protein leads to a decrease of oligomeric stability, further leading to partial dissociation, which was associated with the determination of *M* values smaller than expected [22]. Besides, as mentioned above, Daniel and coworkers also suggested recently that the *M* values of HbLt obtained from different studies give two values in the range of 3.6 to 4.4 MDa [16,20,21]. For this reason, in the current work, *M* determination was performed for two samples of HbGp: one maintained in the oxy form, where the heme is reduced and the iron is in the ferrous state, and one maintained in the cyanomet form, where the heme is oxidized and the iron is in the ferric state but, due to the coordination of cyanide to the iron center, a significant oligomeric stability is achieved. We also tested the HbGp storage time to evaluate the effect of this variable on the *M* of HbGp. The newly determined *M* values for both iron oxidation forms are very close, and the values obtained are significantly more consistent with mass spectrometric data as compared with the old determination [7]. Our results are probably quite relevant for the overall structural determination involving all the subunits of HbGp.

Materials and methods

Protein extraction and purification

HbGp was prepared using freshly drawn blood from worms as reported in the literature [23–25]. The hemoglobin was extracted anesthetizing each animal with ether for a period of 15 to 20 min. An incision was made in the upper part of the animal to avoid the digestive enzymes, and the blood was collected with a Pasteur pipette using sodium citrate as anticoagulant. The collected blood was maintained in ice. The blood sample containing HbGp was prepared by a centrifugation at 4 °C (5000 rpm for 15 min) to eliminate cell debris, followed by an ultrafiltration (molecular weight cutoff of 30 kDa) and ultracentrifugation at 250,000*g* at 4 °C for 6 h. The protein was obtained as a pellet, resuspended in a minimum amount of 0.1 M Tris–HCl buffer (pH 7.0), and stored in the oxy form at 4 °C. Chromatography at pH 7.0 in a Sephadex G-200 column furnished the samples used in our experiments. All concentrations were determined spectrophotometrically using the molar extinction coefficients $\varepsilon_{415nm} = 5.4 \pm 0.8$ $(mg/ml)^{-1}$ cm^{-1} for oxy–HbGp and $\varepsilon_{420nm} = 4.8 \pm 0.6$ $(mg/ml)^{-1}$ cm^{-1} for cyano-met–HbGp [23,24].

Hydrodynamic characterization

The experimental diffusion coefficient (*D*) was obtained by an independent dynamic light scattering experiment using a DynaPro-MS/X device (Protein Solutions) at 20 °C, with proteins in concentration ranging from 0.05 to 1.00 mg/ml in 100 mmol/L Tris–HCl (pH 7.0) containing 50 mmol/L NaCl. The value of *D* was corrected to standard conditions ($D_{20,w}$) and also to infinite protein dilution (0 mg/ml, $D^0_{20,w}$) to avoid effects of viscosity and temperature (see below). *D* is related to the frictional coefficient (*f*) by the following equation:

$$D = \frac{RT}{N_A f}, \tag{1}$$

where *R* is the gas constant, *T* is the absolute temperature, and N_A is the Avogrado's number. *f* for a protein of known Stokes radius (*Rs*) can be obtained by applying the Stokes equation:

$$f = 6\pi\eta Rs. \tag{2}$$

It is possible to estimate the frictional coefficient for a spherical particle (f_0) if one uses the *Rs* for smooth and compact spherical protein (R_0) of molecular mass *M*, which is expressed as

$$R_0 = \left(\frac{3MV_{bar}}{4\pi N_A}\right)^{1/3}, \tag{3}$$

where V_{bar} is the partial specific volume. If one evaluates the ratio between the *f* and f_0, it will give the frictional ratio (f/f_0), which indicates how asymmetric a protein is when compared with a globular protein of the same *M*. f/f_0 is an important factor to study the structure and shape of proteins [26,27]. Through the f_0, one can obtain the maximum diffusion coefficient (D_{sph}) by applying Eq. (1). The f/f_0 value can be obtained directly by either the D_{sph}/D ratio or the Rs/R_0 ratio.

Analytical ultracentrifugation (AUC) experiments were performed in a Beckman Optima XL-A analytical ultracentrifuge. Sedimentation velocity (SV) experiments were carried out in concentrations from 50 to 300 μg/ml in 100 mmol/L Tris–HCl (pH 7.0) containing 50 mmol/L NaCl. HbGp samples with different storage times were tested: 0, 6, and 12 months of storage at 4 °C. The SV experiments were performed at 20 °C using 8000 rpm (AN-60Ti rotor). Curves of absorbance versus cell radius were collected at 232 to 236 nm in intervals of 7 min for each sample. SEDFIT software (version 9.4) was used for calculating the apparent sedimentation coefficient(s) (see below). This value contains interferences caused by temperature, viscosity, and density, so we calculated the standard sedimentation coefficient at infinite dilution (0 mg/ml, $s^0_{20,w}$). SEDNTERP software estimated the sedimentation coefficients at standard conditions (water and 20 °C), called $s_{20,w}$, at each protein concentration from the apparent *s* [26,27]. The $s^0_{20,w}$ was estimated by linear regression. The *M* of a protein can be obtained by the ratio of the sedimentation coefficient to diffusion coefficient using the following equation:

$$M = \frac{sRT}{D(1 - V_{bar}\rho)}. \tag{4}$$