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Further characterization of the subunits of the giant extracellular hemoglobin of *Glossoscolex paulistus* (HbGp) by SDS-PAGE electrophoresis and MALDI-TOF-MS

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ABSTRACT

Further characterization of hemoglobin of *Glossoscolex paulistus* (HbGp) subunits was performed based on SDS-PAGE, size exclusion chromatography (SEC) and MALDI-TOF-MS analysis. SDS-PAGE has shown a total of four linker chains, two quite intense and two of lower intensity. HbGp fractions (I–VI), obtained by size exclusion chromatography (SEC), from oligomeric dissociation at alkaline pH 9.6, were monitored. Fraction I is identical to the whole protein. The monomeric chains *c*, obtained from the trimer *abc* reduction, present four isoforms with *MM* 17,336 Da, 17,414 Da, 17,546 Da and 17,620 Da. Furthermore, the trimer subunit presents two isoforms, T_1 and T_2, with *MM* 51,200 ± 60 and 51,985 ± 50 Da, respectively. Based on SDS-PAGE, the linker chains seem to be distributed along the different fractions of the SEC chromatogram, appearing along the peaks corresponding to fractions I–V. The fraction IV contains, predominantly, trimers with some linkers contamination. The strong interaction of linker chains *L* with the trimers *abc*, makes it difficult to obtain these subunits in pure form. The monomer *d* in fraction VI appears to be quite pure, in agreement with previous studies.

1. Introduction

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 (*MM*), and their oligomeric structure and the crowded and protected heme environment are two of the main factors responsible for the high redox stability. Superoxide dimustase (SOD)-like intrinsic activity, observed for hemoglobins of *Lumbricus terrestris* (HbLt) and of *Arenicola marina* (HbAm), is another important factor [1,4]. 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 [5,6].

Moreover, a strong motivation to study these giant hemoglobins is related to their potential use in medicine as blood substitutes. Studies are presently underway to test and validate the use of *A. marina* hemoglobin (HbAm) in this direction [4,7]. 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 [4,7]. Besides that, 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 quaternary structure of this class of hemoglobin is constituted by two superimposed hexagonal arrays of twelve spherical subunits, the hexagonal bilayer (HBL). The extracellular hemoglobins are constituted of four subunits, containing heme group, chains *a*, *b*, *c* and *d*. The subunits *a*, *b* and *c* are linked by disulfide bonds forming the trimer (*abc*) and subunit *d* remains in the monomeric form [8]. Additional structural components named linker chains do not have the heme group. These subunits are probably related to the maintenance of the stability of the oligomeric structure [4].

Recent partial characterization of oxy-HbGp (giant extracellular hemoglobin of *Glossoscolex paulistus*) molecular mass by matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) confirmed the similarity of its subunits to those of orthologous proteins of this class, mentioned above [9]. This characteristic multisubunit content confers to the whole protein a double-layered hexagonal oligomeric structure [10,11]. The *MM* of HbGp has been re-examined recently by analytical ultracentrifugation (AUC), giving results of *MM* of approximately 3600 ± 100 kDa and 3700 ± 100 kDa for oxy- and cyanomet-forms, respectively, at pH 7.0 [12]. Studies based on AUC, dynamic light scattering (DLS) and fluorescence emission spectroscopy show that the oxy-HbGp at pH 7.0 remains in its full oligomeric state, while at pH 9.0 it is partially dissociated [12,13].

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It is worthy of notice that HbGp belongs to the same class of hemoglobins as *L. terrestris* (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. Daniel et al. have argued that the *MM* of HbLt could vary between 3.6 and 4.4 MDa [14,15]. 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, $(abcd)_4L_3$ [14]. On the other hand, Vinogradov et al. [16] have proposed a model for HbLt, where 1/12 of the whole molecule is given by $(abcd)_3L_3$, 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.

Recent work by Royer et al. on the crystal structure of HbLt [17,18] has suggested that the Vinogradov model is very consistent with the crystal structure. Besides that, very recent preliminary work on the crystal structure of HbGp [19] 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. 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 this work, MALDI-TOF-MS analysis of the extracellular hemoglobin of *G. paulistus*, HbGp, in the met- and cyanomet-forms, has been performed to further characterize the *MM* of the subunits of this protein, at pH 7.0. Moreover, all fractions obtained by size exclusion gel filtration chromatography (SEC) of oxy-HbGp at pH 9.6 were characterized by polyacrylamide gel electrophoreses (SDS-PAGE) and MALDI-TOF-MS. The detailed studies of the molecular masses of HbGp subunits can contribute to the elucidation of the stereochemistry and of the quaternary structure of this interesting HBL hemoglobin.

2. Materials and methods

2.1. Protein extraction and purification

G. paulistus annelid is prevalent in sites near Piracicaba and Rio Claro cities in the state of São Paulo, Brazil. The HbGp was prepared using freshly drawn blood from worms. HbGp solution was centrifuged at 2500 rpm, 15 min at 4 °C. Then the sample was filtered (Mw cut-off 30 kDa) and centrifuged at 250,000 × *g* at 4 °C during 3 h. The pellet was resuspended in a minimum amount of 0.1 mol/L Tris–HCl buffer at pH 7.0. Chromatography at pH 7.0 in a Sephadex G-200 column furnished the samples used in the experiments [13,20,21]. All concentrations were determined spectrophotometrically in a UV-SHIMADZU 1601PC spectrophotometer, using the appropriate molar absorption coefficients [13,22]. The final protein concentration corresponding to our stock solution was in the range of 15–30 mg/mL in Tris–HCl 0.1 mmol/L buffer, pH 7.0. In order to obtain the oxidized met-HbGp, an excess of 5-fold potassium ferricyanide relative to heme is added followed by 2 h incubation. To obtain the cyanomet-HbGp form, the met-HbGp was further incubated with a 5-fold excess of potassium cyanide relative to the heme concentration. All concentrations were determined spectrophotometrically using the molar extinction coefficients $\varepsilon_{415\,nm} = 5.5 \pm 0.8\,(mg/mL)^{-1}\,cm^{-1}$ for oxy-HbGp, $\varepsilon_{402\,nm} = 4.1 \pm 0.7\,(mg/mL)^{-1}\,cm^{-1}$ for met-HbGp and $\varepsilon_{420\,nm} = 4.8 \pm 0.5\,(mg/mL)^{-1}\,cm^{-1}$ for cyanomet-HbGp [13,23].

2.2. Size exclusion chromatography (SEC)

The size exclusion chromatography (SEC) was performed with a Superdex 200 TM prep grade column coupled to an ÄKTA prime plus system (GE). The system was equilibrated with 100 mmol/L Tris–HCl buffer, pH 7.0 and flow rate 1 mL/min, the sample concentrations were approximately 18 mg/mL, and the elution sample volume was 1 mL. The elution solution was the same buffer used for column equilibration. The elution was monitored at a wavelength of 280 nm in the instrument, and, additionally, at 415 nm in a Shimadzu UV-1601PC spectrophotometer. The use of these two wavelengths is very convenient for monitoring hemoglobin samples, since at 280 nm a direct measurement of protein (absorption is due mainly to the aromatic aminoacids) is made, while at 415 nm the absorption is due to the hemoglobin heme groups. In this way, simultaneous monitoring of protein and heme content can be achieved. Fractions of the subunits of the hemoglobin of *G. paulistus* were pooled together, stored at 4 °C, and characterized by electrophoresis SDS-PAGE. Samples for MALDI-TOF-MS were further dialyzed against a 5 mmol/L Tris–HCl buffer to reduce the salt concentration. All experiments were performed at 25 °C.

2.3. Gel electrophoresis

The molecular masses of the protein subunits were determined by SDS-PAGE from the fractions collected from the SEC experiments. The percentage of acrylamide used was of 15% at pH 8.6. Samples were run with Tris–HCl 25 mmol/L and glycine 192 mmol/L buffer, pH 6.8, at a constant applied voltage of 140 V. Gels were stained with Coomassie Brillant R-250 from Bio-Rad. The electrophoresis experiments were always made in two plates, simultaneously, one without β-mercaptoethanol and the other one in the presence of the reducing agent. Molecular masses markers were used to determine the apparent molecular mass of the subunits and supplied by Page Ruler TM Unstained Protein Ladder of Fermentas. Relative molecular masses of separated proteins were estimated using protein standards run in parallel in the same gels.

2.4. Matrix-assisted laser desorption/ionization time-of-flight/mass spectrometry – MALDI-TOF-MS

The protein fractions isolated by size exclusion chromatography (SEC) and oxy-, met- and cyanomet-integral HbGp oxidation forms, were subjected to analyses on a matrix-assisted laser desorption time-of-flight mass spectrometer. The protein solutions were extensively dialyzed to reduce the buffer (salt) concentration to 5 mmol/L, followed by a further dilution to about 1–3 mg/mL, which is the protein concentration used in MALDI-TOF-MS experiments.

The 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid) was obtained from Aldrich. 2-Mercaptoethanol, cytochrome *c* (from Bovine Heart) and bovine serum albumin (BSA) were obtained from Sigma. The laser desorption matrix material (sinapinic acid) was dissolved in 0.5% trifluoroacetic acid, 50% acetonitrile:water. Samples of the whole native HbGp and the isolated fractions of HbGp were mixed in ratios of 1:5, 1:10 and 1:20 (v/v) with a saturated solution of sinapinic acid [9].

The greater dilution with the matrix was generally made for the whole protein sample which was more concentrated (3.0 mg/mL of protein). One microliter of this mixture (sample/matrix) was spotted onto a MALDI plate and analyzed in MALDI-TOF-MS. Analysis were performed, randomly, in linear, positive ion mode in an Etthan MALDI-TOF mass spectrometer (Amersham Bioscience) using an acceleration voltage of 20 kV. Each spot was analyzed twice, accumulating spectra composed of approximately 200 laser shots in total and the resulting spectra were analyzed by Ettan Evaluation software (Amersham Bioscience). The instrument was calibrated using external cytochrome *c* and BSA standards.

The reported molecular masses were obtained as averages of several individual experiments and taking into account the values obtained for single protonated and double protonated molecular species.

3. Results and discussions

3.1. Size exclusion chromatography (SEC) and electrophoresis – SDS-PAGE

In Fig. 1A the chromatogram of a SEC experiment for oxy-HbGp 18 mg/mL, incubated at pH 9.6 for 15 h, followed by re-acidulation back to pH 7.0 prior to application to the column, is shown. Chromatography was performed at pH 7.0. Several species are observed in equilibrium, and in the chromatogram six fractions (indicated by I–VI) were selected and pooled together for further analysis. Detailed assignment of these fractions can only be made based on SDS-PAGE and MALDI-TOF-MS data described later on. However, on the basis of our previous work [9], and on the ratio of heme to protein content, as monitored by the absorbance ratio A_{415}/A_{280} (Fig. 1B), it is possible to make some associations. The first fraction I corresponds to a protein fraction very close to the whole un-dissociated native HbGp eluting in the dead volume, the fourth fraction IV is, predominantly, due to the contribution of the trimer *abc*, and the sixth fraction VI corresponds to the monomeric subunit *d* in a very pure state as observed in previous MALDI-TOF-MS [9] and ultracentrifugation studies [24]. The remaining fractions, II, III and V, have been subjected to further studies by other techniques to

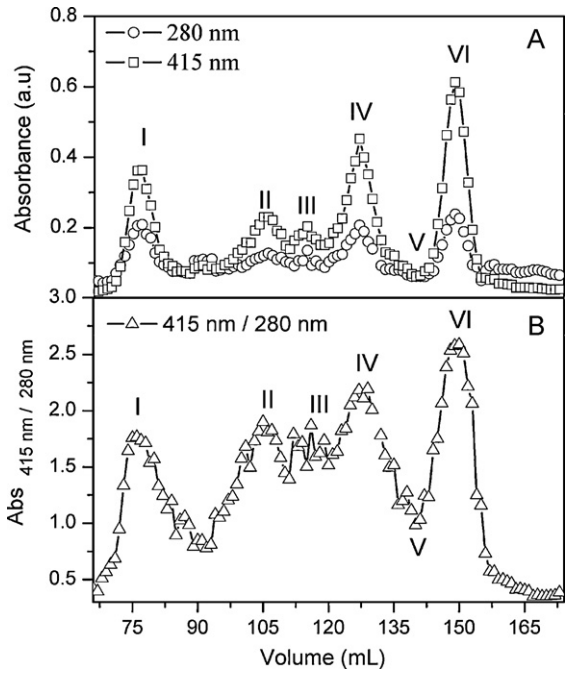

Fig. 1. (A) Superdex 200 gel filtration chromatograms of oxy-HbGp 18.0 mg/mL, in Tris–HCl 0.1 mol/L, pH 7.0, at 25 °C. Detection wavelengths are 280 nm (circles) and 415 nm (squares). (B) Ratio of absorbances at 415 nm and 280 nm. Roman numerals (I–VI) correspond to different species obtained for alkaline dissociation of HbGp, which were pooled and concentrated for further analysis.

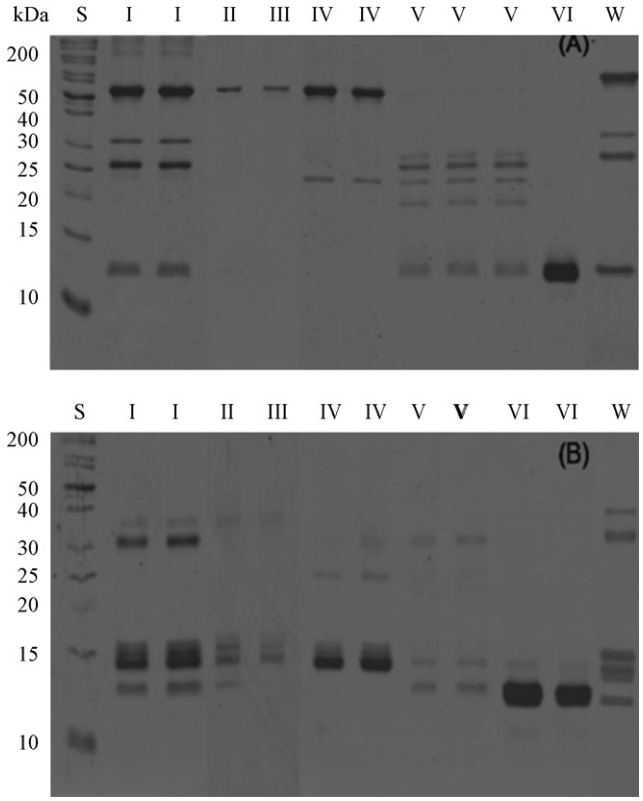

Fig. 2. SDS-polyacrylamide gel electrophoresis of fractions recovered from gel filtration in Superdex 200 column of oxy-HbGp. The gel concentration was 15% in 25 mmol/L Tris–HCl, 192 mmol/L glycine, pH 8.3, and stained with Coomassie Blue R-250. Molecular masses of standard proteins were of 200, 50, 40, 30, 25, 20, 15 and 10 kDa and are indicated. The slot (S), (W) corresponds to the standard masses and whole protein (HbGp), respectively, in gels (A) and (B). (A) Gel electrophoresis in the absence of β-mercaptoethanol and (B) with the reducing agent. Roman numerals correspond to the different species obtained for alkaline dissociation and gel filtration as shown in Fig. 1A.

improve our analysis. It is worthy of mentioning, that fraction V is quite rich in linker subunits, as will be seen below. This is expected on the basis of the very low ratio of absorbances at 415 nm and 280 nm (Fig. 1B), implying lower heme content. In fact, the choice of this fraction was mainly motivated to evaluate the content and nature of the linker chains.

All fractions I–VI from the chromatogram in Fig. 1A were monitored by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), in the absence or presence of β-mercaptoethanol. Fig. 2A shows the results of the electrophoresis of HbGp fractions obtained from the SEC experiment described in Fig. 1A, in the absence of the reducing agent. The first lane contains the protein standards of *MM* in the range from 10 to 200 kDa. Lanes 2–3 and 6–7 correspond, respectively, to fractions I and IV in Fig. 1A. They share a common band at 47.8 ± 0.5 kDa (see Table 1) that is assigned to the trimer *abc*, observed in previous MALDI-TOF-MS experiments with a mass around 52 kDa [9]. Fraction I also contains contributions from linkers (32.4 ± 0.3 and 27.3 ± 0.5 kDa) and monomers (12.3 ± 0.3 kDa, see Table 1). It is worthy of notice, that fraction I is, probably, the whole HbGp oligomer. This can be seen by comparison of fraction I (lanes 2–3) with the whole HbGp eletrophoresis profile shown in the last lane (denoted as W) of Fig. 2A (see also the last column of Table 1). The fraction IV, on the other hand, contains a significant amount of trimers *abc* and, additionally, a small amount of a different linker chain at 24.1 ± 0.3 kDa (see Table 1). Its content is, predominantly, associated to the trimer *abc*. The fraction V in the chromatogram in Fig. 1A shows several relatively weak peaks in the *MM* range between 20 and 30 kDa, which are, probably, associated to the linker chains and some small contamination of monomers *d* and trimers *abc*. Fractions II and III are shown, respectively, in lanes 4 and 5. Since their protein concentrations are lower, as compared to the other peaks in the chromatogram of Fig. 1A, the intensity of the corresponding bands are smaller. Finally, the fraction VI is, probably, the most intense and pure band in the chromatogram of Fig. 1A, corresponding to the monomer *d* with a *MM* of 12.3 ± 0.3 kDa (Table 1).

In Fig. 2B the results of electrophoresis for the samples with β-mercaptoethanol are presented. Here again, as in Fig. 2A, the lanes 2–3 and 6–7 correspond to fractions I and IV, respectively, in the chromatogram of Fig. 1A. For fraction I the band at 47.8 ± 0.5 kDa is not observed in this case (see Table 1, lower line marked either with an asterisk or with the absence of numbers), indicating that the trimer disulfide bonds were reduced. Besides that, three additional bands are observed in the gel above the 12.3 kDa monomeric band (Table 1, lower line marked either with an asterisk or with the absence of numbers). They are due to the contribution of the monomeric subunits *a*, *b* and *c* produced upon disulfide bond reduction of the trimer. This reduction of the trimer is also clearly observed for the fraction IV in the gel (lanes 6–7). The contributions of the monomeric subunits *a*, *c* and *b*, correspond to *MM* of 16.2 ± 0.2 kDa, 14.9 ± 0.1 kDa and 14.3 ± 0.3 kDa, respectively (Table 1, lower lines marked with an asterisk). The fraction V (lanes 8 and 9, Fig. 2B) presents several subunits, such as the trimer *abc* and its monomers obtained upon reduction, and the monomer *d*, with similar masses as those for the fraction IV (Table 1). However, besides the linker at 28.8 ± 0.5 kDa that is almost coincident with one of the two linkers (at 32.4 ± 0.3 and 27.3 ± 0.5 kDa), observed for the whole native protein, two additional linkers are detected at 26.5 and 23.7 kDa (see Fig. 2B and Table 1).

It is important to notice, that in the lanes 8 and 9 (Fig. 2A and B) four bands are noticed, that could be associated to different linker chains. The most intense, the second larger *MM*, at 27.3 kDa, seems to correspond to the lower *MM* linker band

Table 1
Molecular masses (kDa) of *Glossoscolex paulistus* hemoglobin (HbGp) subunits, obtained from SDS-PAGE electrophoresis in the absence and presence of β-mercaptoethanol.

Subunits	Fractions (masses (kDa))						
	I	II	III	IV	V	VI	W[b]
Trimer (*abc*)	47.8 ± 0.5	48.3 ± 0.4	48.3 ± 0.4	47.8 ± 0.5	47.8 ± 0.5	–	49.1 ± 0.6
	–	–	–	–	–	–	–
Linker	32.4 ± 0.3	32.7 ± 0.3	–	–	–	–	33.1 ± 0.5
	32.5 ± 1.2[a]	–	–	–	–	–	34.5 ± 1.0[a]
	27.3 ± 0.5	27.2 ± 0.4	27.2 ± 0.4	–	28.8 ± 0.5	–	27.5 ± 0.5
	30.9 ± 0.4[a]	30.4 ± 0.4[a]	30.4 ± 0.4[a]	30.1 ± 0.3[a]	30.9 ± 0.4[a]	–	31.6 ± 0.5[a]
	–	–	–	–	26.5 ± 0.5	–	–
	–	–	–	26.2 ± 0.2[a]	25.9 ± 0.2[a]		–
	–	–	–	24.1 ± 0.3	23.7 ± 0.5	–	–
	–	–	–	24.1 ± 0.3[a]	23.1 ± 0.2[a]		–
Monomer (*a*)	–	–	–	–	–	–	–
	16.2 ± 0.1[a]	16.1 ± 0.2[a]	16.1 ± 0.1[a]	16.2 ± 0.2[a]	15.9 ± 0.6[a]	–	15.5 ± 0.7[a]
Monomer (*c*)	–	–	–	–	–	–	–
	14.9 ± 0.2[a]	14.8 ± 0.2[a]	14.8 ± 0.2[a]	14.9 ± 0.1[a]	15.0 ± 0.2[a]	15.0 ± 0.2[a]	14.1 ± 0.4[a]
Monomer (*b*)	–	–	–	–	–	–	–
	14.4 ± 0.4[a]	14.0 ± 0.4[a]	14.0 ± 0.4[a]	14.3 ± 0.3[a]	13.6 ± 0.4[a]		13.3 ± 0.5[a]
Monomer (*d*)	12.3 ± 0.3	12.6 ± 0.3	–	–	12.3 ± 0.2	12.3 ± 0.3	11.7 ± 0.5
	12.3 ± 0.7[a]	11.6 ± 0.7[a]	–	–	12.3 ± 0.7[a]	12.3 ± 0.7[a]	11.4 ± 0.3[a]

The roman numerals correspond to the peaks (fractions) obtained through gel filtration as shown in Fig. 1A.
[a] The samples in the presence of β-mercaptoethanol. The errors were obtained by an average of data obtained from several runs.
[b] This column corresponds to the whole native HbGp.

observed for the whole protein and fraction I. However, it is not safe to infer only based on SDS-PAGE electrophoresis, which are exactly the bands corresponding to the linker chains L_1, L_2, L_3 and L_4 that are expected for HbGp, in total analogy with the reported data for HbLt [5,6]. The reason for this is the fact that SDS-PAGE electrophoresis results can be considered only qualitatively, not as quantitative data. The mass values estimated by electrophoresis are very dependent on the protein migration properties through the gel used in SDS-PAGE. The migration in the gel is strongly dependent upon the protein hydrodynamic properties, shape and surface charge characteristics. The main difference in subunits *MM* based on the electrophoresis is observed for the monomers and trimers. Any factor that affects the shape of the subunits will interfere with their migration in the electrophoresis gel. For this reason, all the molecular masses estimates based on SDS-PAGE eletrophoresis have only a qualitative meaning, serving mostly for comparisons. Besides that, SDS-PAGE is certainly very important for the assignment of the SEC chromatographic peaks.

The fraction V of the chromatogram in Fig. 1A is expected to present a high amount of linker subunits due to its low absorption at 415 nm associated to a low heme content (see Fig. 1B). However, it seems that the linker subunits are distributed differently and spread over the different fractions eluted in the chromatography of Fig. 1A. Some of them prefer to remain associated either to a fraction close to the whole oligomer (fractions I and II in Fig. 1A), while some are eluted with the trimer in the chromatographic process (fractions IV and V in Fig. 1A), as observed by SDS-PAGE (Fig. 2A and Table 1).

Observing Fig. 2A and B, it is clearly seen that the migration of HbGp subunits, in the presence of β-mercaptoethanol, is somewhat different, through the gel, producing, in general, a less resolved pattern. This could be associated to some additional unfolding of the protein induced by the reducing agent as compared to the SDS-PAGE performed in its absence. For this reason, a small difference between the masses presented in Table 1 for the same species, in the absence and the presence of reducing agent (lower lines marked with an asterisk), is observed.

3.2. MALDI-TOF-MS

3.2.1. Whole protein

In Fig. 3 the MALDI-TOF-MS spectrum of a solution of cyanomet-HbGp, at pH 7.0, using sinapinic acid as a matrix in the positive ion

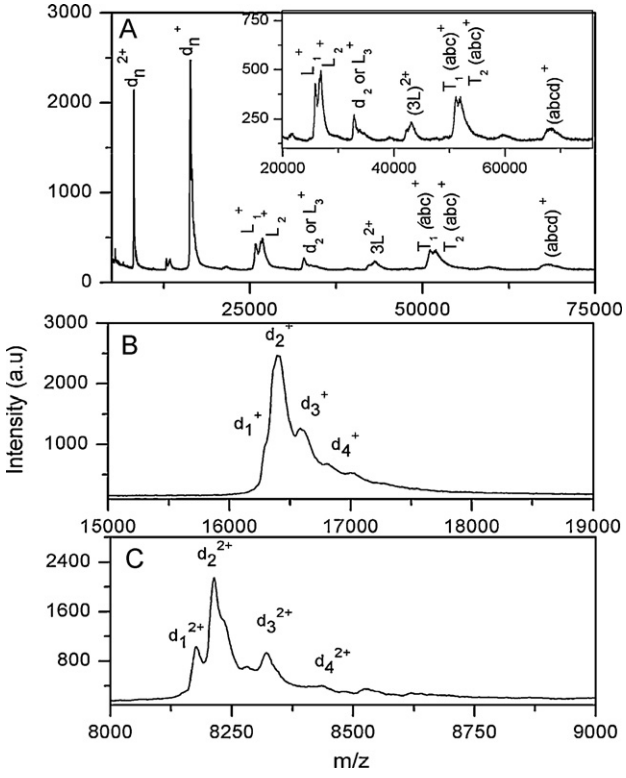

Fig. 3. MALDI-TOF-MS spectrum of cyanomet-HbGp (A) at pH 7.0 without β-mercaptoethanol. The insert shows the expanded ordinate, corresponding to the intensity in arbitrary units, highlighting the trimer and linkers peaks; (B) the expanded region for the mono-protonated monomer d^+ from 15,000 to 19,000 Da; and (C) the expanded region for the double-protonated monomer d^{2+} from 8000 to 9000 Da. d_1, d_2, d_3, and d_4 correspond to monomer *d* isoforms.

Table 2
Molecular masses (Da) of *Glossoscolex paulistus* hemoglobin (HbGp) subunits, at different indicated iron oxidation states, obtained from MALDI-TOF-MS.

Subunits	Oxy-HbGp	Oxy-HbGp[a]	Cyanomet-HbGp	Cyanomet-HbGp[a]	Met-HbGp	Met-HbGp[a]
d_1	16,370	16,360	16,350 ± 15	16,345 ± 20	16,335 ± 20	16,360 ± 4
d_2	16,415 ± 10	16,445 ± 20	16,410 ± 15	16,440 ± 30	16,412 ± 20	16,495 ± 20
d_3	16,650 ± 40	16,620	16,615 ± 30	16,620	16,630 ± 10	–
d_4	16,850 ± 40	16,820	16,835 ± 35	16,820	16,795 ± 50	–
b	–	16,480	–	16,500	–	16,460 ± 40
c_1	–	17,330 ± 15	–	17,320 ± 5	–	17,385 ± 10
c_2	–	17,410	–	17,413 ± 20	–	17,410 ± 40
c_3	–	17,546	–	17,548 ± 10	–	17,558 ± 15
c_4	–	17,620	–	17,620 ± 5	–	–
a	–	18,245 ± 20	–	18,245 ± 20	–	18,267 ± 20
L_1	25,780 ± 30	25,870 ± 50	25,860 ± 30	25,870 ± 15	25,817 ± 20	25,940 ± 100
L_2	26,750 ± 80	26,720 ± 80	26,815 ± 20	26,870 ± 60	26,885 ± 40	26,875 ± 100
$2d$[b]	–	33,870	–	33,710	–	33,985 ± 100
L_4	–	–	–	–	–	–
L_3 or $2d$[c]	32,870	32,900	32,850	32,910	32,865 ± 80	32,890 ± 80
$2c_2$	–	34,700	–	34,620	–	34,635 ± 20
T_1 (abc)	51,200	–	51,140	–	51,220 ± 100	–
T_2 (abc)	51,985	51,630	51,940	51,400	52,025 ± 100	51,915 ± 70
$abcd$	68,400	69,280	68,300	68,270	68,275 ± 60	69,675 ± 35

[a] Samples in the presence of β-mercaptoethanol.
[b] The value of mass corresponds very closely to the dimer of the monomer isoform d_4.
[c] The value of mass corresponds very closely to the dimer of the monomer isoform d_2.

mode, is shown without β-mercaptoethanol. In agreement with previous studies [9], Fig. 3A shows an intense peak centered around 16.3 kDa, corresponding to the monomer *d* subunit, which is consistent with its relatively easy ionization. Besides that, characteristic peaks due to the trimer *abc*, linkers chains, and tetramer *abcd*, with different ionization degrees, are also noticed in the spectrum. The *MM* observed for d^+ subunits were 16,350 ± 15 Da, 16,410 ± 15 Da, 16,615 ± 30 Da and 16,835 ± 35 Da, corresponding, respectively, to single protonation of isoforms d_1, d_2, d_3, and d_4 (Fig. 3B and Table 2). In Fig. 3C the monomer d^{2+} isoforms, double protonated, are shown and a higher definition of the individual peaks is observed as compared to Fig. 3B. The monomer isoform masses in Table 2 are an average of the values obtained from the spectra in Fig. 3B and C. The *MM* of 25,860 ± 30 Da and 26,815 ± 20 Da are attributed to linker chains L_1 and L_2 mono-protonated species (Table 2). The observed *MM* values for the peptide chains of cyanomet-HbGp in the present work are in agreement with the values observed previously for oxy-HbGp [9,24].

As can be noticed from Fig. 3A and Table 2, the trimer *abc* masses of 51,140 Da and 51,940 Da, and the tetramer *abcd* mass of 68,300 Da, were also observed, implying the existence of, at least, two isoforms for the trimeric subunit, all of them mono-protonated. The dimer of monomers, $(2d_2)^+$, with a mass of 32,850 Da is, probably, superposed to the L_3 linker. The fourth linker (L_4) is not clearly observed by MALDI-TOF-MS data, suggesting that this subunit is, probably, less abundant and the contribution of this species is not observed [5,6]. The linker at 25,860 ± 30 Da could also be overlapped with the double protonated trimer $(abc)^{2+}$ [5,6]. The small intensity of the tetrameric subunit peak is, probably, associated to its low ionization, even smaller than that for the trimer [9]. The trimer subunit isoforms are associated to the variations in composition of chains *c*, which are constituted by several isoforms, as will be shown below. Literature reports regarding HbLt have also shown that chains *a* are constituted by different isoforms [5,6]. Our present results show that in the case of HbGp the subunits presenting several isoforms are *d* and *c* chains.

The MALDI-TOF-MS spectrum for cyanomet-HbGp in the presence of β-mercaptoethanol is shown in Fig. 4. Analysis of the inset of Fig. 4A, focusing the range from 30,000 to 38,000 Da, shows three resolved peaks with the following masses: (1) 32,910, (2) 33,710, and (3) 34,620 Da. Considering their half value, masses of 16,405, 16,855 and 17,320 Da are obtained. Based on the masses given in Table 2, these peaks could correspond to the following species: (1) dimer of monomers species, ($2d_2$), or linker L_3, (2) dimers of monomer species ($2d_4$) and (3) dimers of monomers (2*c*). In Fig. 4B and C the expanded regions for mono-protonated and double protonated monomers are shown, respectively. These figures show

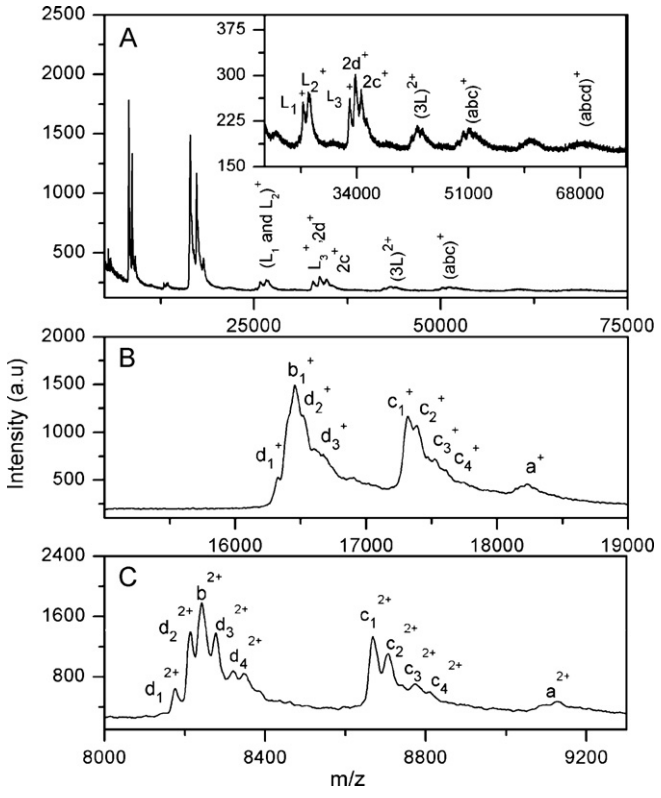

Fig. 4. MALDI-TOF-MS spectrum of cyanomet-HbGp (A) at pH 7.0 with β-mercaptoethanol. The insert shows the expanded ordinate, corresponding to the intensity in arbitrary units, highlighting the trimer and linkers peaks; (B) the expanded region for the mono-protonated monomer d^+ from 15,000 to 19,000 Da; and (C) the expanded region for the double-protonated monomer d^{2+} from 8000 to 9000 Da. d_1, d_2, d_3, and d_4 correspond to monomer *d* isoforms. c_1, c_2, c_3, and c_4 correspond to monomer *c* isoforms, obtained upon reduction of trimer *abc* disulfide bonds.

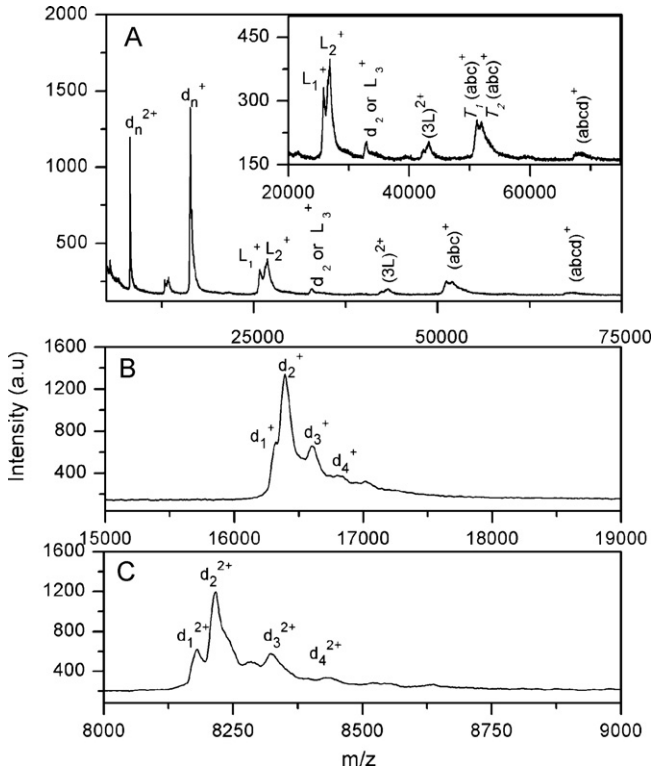

Fig. 5. MALDI-TOF-MS spectrum of met-HbGp (A) at pH 7.0 without β-mercaptoethanol. The insert shows the expanded ordinate, corresponding to the intensity in arbitrary units, highlighting the trimer and linkers peaks; (B) the expanded region for the mono-protonated monomer d^+ from 15,000 to 19,000 Da; and (C) the expanded region for the double-protonated monomer d^{2+} from 8000 to 9000 Da. d_1, d_2, d_3, and d_4 correspond to monomer *d* isoforms.

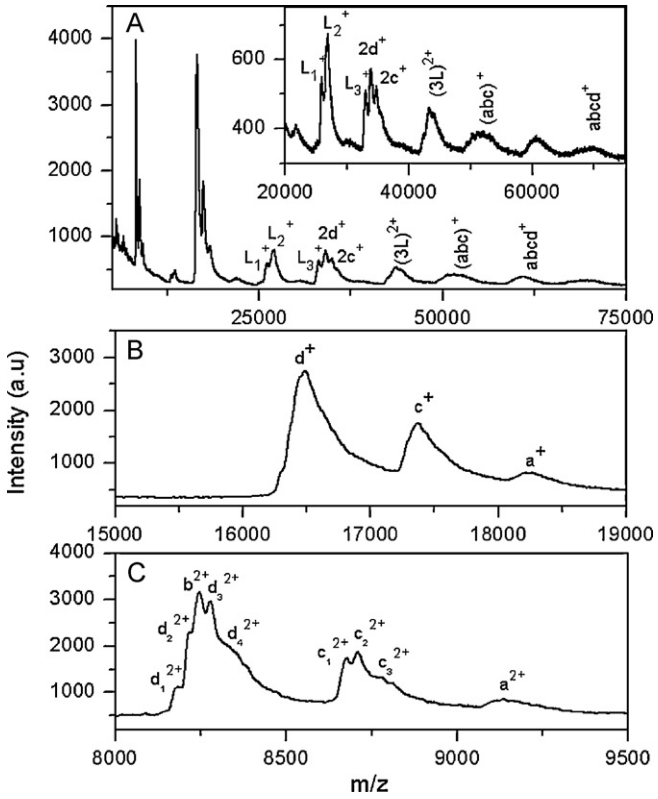

Fig. 6. MALDI-TOF-MS spectrum of met-HbGp (A) at pH 7.0 with β-mercaptoethanol. The insert shows the expanded ordinate, corresponding to the intensity in arbitrary units, highlighting the trimer and linkers peaks; (B) the expanded region for the mono-protonated monomer d^+ from 15,000 to 19,000 Da; and (C) the expanded region for the double-protonated monomer d^{2+} from 8000 to 9500 Da. d_1, d_2, d_3, and d_4 correspond to monomer *d* isoforms. c_1, c_2, c_3, and c_4 correspond to monomer *c* isoforms, obtained upon reduction of trimer *abc* disulfide bonds.

clearly the multiple isoforms for monomeric chains *d* and *c*. The *MM* of subunits *c* are 17,320 ± 5 Da, 17,413 ± 20 Da, 17,548 ± 10 Da and 17,620 ± 5 Da (Table 2), corresponding to isoforms c_1, c_2, c_3, and c_4, respectively. The *MM* values of subunit *d* isoforms are shown in Fig. 3B and C, and the values of the other chains *b* and *a*, are, respectively, 16,500 and 18,245 ± 20 Da (Table 2). Since monomeric chain *a* has a low intensity due to an inefficient ionization, apparently a single mass value is obtained. On the other hand, the chain *b* is superposed with the chains *d* and here again a single unique chain is observed.

Figs. 5 and 6 show, respectively, the MALDI-TOF-MS spectra of met-HbGp 3.0 mg/mL, in the absence and the presence of β-mercaptoethanol. The general behavior observed for this species was similar to that for the other two forms, oxy- and cyanomet-HbGp. However, the peaks resolution, in the region between 15,000 Da and 19,000 Da, for the met-form, in the presence of β-mercaptoethanol, is lower as compared to cyanomet-HbGp (Fig. 6B and C).

In summary, our results suggest that HbGp subunits are very similar in mass to HbLt, but the chains *c* and *d* of HbGp display a total of four isoforms, while in HbLt the chains having several isoforms are *d* and *a*. Our present results correspond to a further characterization of HbGp subunit masses. However, more detailed information is still necessary, especially regarding the linker chains, which are not so easily isolated in the pure form as seen both in the gel filtration and eletrophoretic experiments. Besides that, work is in progress to obtain the subunits sequences. This is certainly an important and interesting matter for future work. Overall, globin chains of HbGp are quite similar to those of HbLt, as expected, presenting several isoforms. Their relevance for the hemoglobin functioning remains another interesting matter for future research.

3.2.2. Isolated protein fractions

In Table 3 the masses corresponding to each protein fraction, obtained from the gel filtration of the whole oxy-HbGp exposed to pH 9.6 (Fig. 1A), are shown. They can be compared to the first column in Table 2, which corresponds to the whole native oxy-HbGp. The fraction I displays a similar composition as the whole protein, suggesting the preservation of the native HbGp structure. The contribution of the tetrameric species $(abcd)^+$ is not observed in this fraction, and this could be associated to the smaller sample concentration as compared to the native HbGp. The presence of a third linker, L_3, which is superposed to the dimer of monomers, *2d*, is also not clearly observed. Moreover, the masses of the monomeric and trimer isoforms are very similar. It is worthy of notice, that for the two linker chains observed in fraction I, the masses are higher than those reported for the native protein (around 100 Da). Fraction II was analyzed, in the presence and the absence of β-mercaptoethanol. For this fraction, the addition of the reducing agent leads to some broadening of the monomeric peak in the spectrum precluding the resolution of the four isoforms (see Table 2 for the whole oxy-HbGp). Only the more intense d_2 isoform gave a precise mass value (16,470 ± 14 Da). The mass values of the two linker chains L_1 and L_2 seem to be also somewhat higher (200 Da) in the presence of the reducing agent. The mass of the tetramer also presents the same trend (1 kDa). Based on the masses presented in Table 3, fractions II and III are quite similar and seem to be formed by the same subunits as the whole native protein.

The fraction IV is expected to be rich in trimers *abc*. As can be seen from Table 3 it is quite pure, presenting only three peaks: one at 16,430 Da, corresponding to monomer isoform d_2, some con-

Table 3
Molecular masses (Da) of *Glossoscolex paulistus* hemoglobin (HbGp) subunits, obtained from MALDI-TOF-MS, for the fractions obtained in the gel filtration chromatography as shown in Fig. 1A.

Subunits	Fractions (masses in Da)					
	I	II	II[a]	III	IV	VI
d_1	16,350	16,332 ± 21	–	16,334 ± 23	–	16,330 ± 10
d_2	16,423 ± 18	16,406 ± 19	16,470 ± 14	16,410 ± 14	16,430	16,405 ± 17
d_3	16,638 ± 25	16,605 ± 37	–	16,627 ± 19	–	16,627 ± 15
d_4	16,860	16,825 ± 20	–	16,832 ± 33	–	16,826 ± 11
b	–	–	–	–	–	–
c_1	–	–	17,365 ± 35	–	–	–
c_2	–	–	–		–	––
c_3	–	–	17,556 ± 20		–	–
c_4	–	–	17,750 ± 50		–	–
a	–	–	18,265 ± 35	–	–	–
L_1	25,970 ± 25	25,897 ± 84	26,115 ± 50	25,940	25,820	–
L_2	26,860 ± 60	26,797 ± 26	27,017 ± 4	26,630 ± 90	26,620 ± 100	–
$2d$[b]	–	–	34,045	–	–	33,885 ± 200
L_4	–	–	–	–	–	–
L_3 or $2d$[c]	–	33,270 ± 100	33,190	33,180	–	32,740 ± 200
$2c_2$	–	–	34,820	–	–	–
$3d$						49,700
T_1 (abc)	51,230 ± 30	51,200 ± 45	–	51,080 ± 45	–	–
T_2 (abc)	51,940 ± 60	51,950 ± 40	51,590	51,875 ± 100	52,270	–
$abcd$	–	68,280	69,260	68,260	–	–

The roman numerals correspond to the peaks (fractions) obtained through gel filtration as shown in Fig. 1A.
[a] Samples in the presence of β-mercaptoethanol.
[b] The value of mass corresponds very closely to the dimer of the monomer isoform d_4.
[c] The value of mass corresponds very closely to the dimer of the monomer isoform d_2.

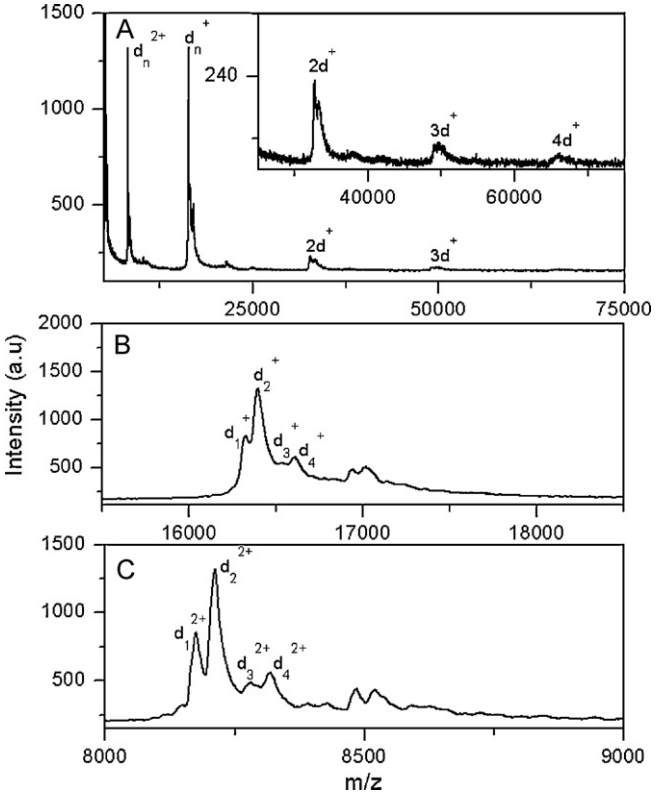

Fig. 7. MALDI-TOF-MS spectrum of fraction VI, obtained from native HbGp in the oxy-form, through chromatography on a Superdex 200 column as shown in Fig. 1, and corresponding to the pure monomer *d*: (A) whole MS spectrum; the insert shows the expanded ordinate, corresponding to the intensity in arbitrary units, highlighting the trimer and linkers peaks; (B) the expanded region of masses from 16,000 to 18,500 Da for the mono-protonated monomer d^+; and (C) the expanded region from 8000 to 9000 Da for the double-protonated monomer d^{2+}. d_1, d_2, d_3, and d_4 correspond to monomer *d* isoforms.

tamination at 25,820 Da, 26,620 ± 100 Da, which can be assigned to linker chains L_1 and L_2, respectively. The highest contribution in this fraction is the peak at 52,270 Da, assigned to the trimer *abc*. This peak is quite intense and the two isoforms are not well resolved. In our previous work [9], it was noticed that the monomers are easily ionized, while the trimers do not behave in the same way. In fact, the fraction associated to the trimers is not very stable under conditions of low salt concentration, required for MALDI-TOF experiments, and tend to precipitate upon long standings. Our results regarding the fraction IV are consistent with gel filtration data as well as the electrophoresis analysis. Moreover, no contributions of tetramer *abcd* and dimer of monomers *2d* are observed for this fraction. The contamination of fraction IV with linkers is observed in the gel presented in Fig. 2A, and supports the observation of the strong interaction of linkers with the *abc* trimer.

Our data show that fraction VI corresponds to pure monomer *d*, where only the contributions of its several mono-protonated isoforms, in the mass range from 15,000 Da to 19,000 Da, dimers of monomers in the range between 30,000 and 35,000 Da, and trimers of monomers around 49,700 Da (Fig. 7), are observed, suggesting that peak VI in Fig. 1A corresponds to monomer *d* in a very pure form. These results are also consistent with the observed SDS-PAGE electrophoresis data (see Fig. 2A).

The fraction V, expected to be quite abundant in several linker chains, was very diluted and its low concentration was inadequate for mass spectrometry analysis.

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 study, further characterization of *G. paulistus* hemoglobin was performed, through the use of size exclusion chromatography, SDS-PAGE electrophoreses and MALDI-TOF-MS. The gel filtration chromatography of HbGp, submitted to alkaline oligomeric dissociation, is capable to produce the separation of several protein subunits. The electrophoresis analysis of the fractions produced in

SEC experiments, indicate that HbGp is composed by many subunits. Moreover, our present results show an important progress in the HbGp subunits characterization, especially regarding the difficulties encountered to separate completely the linker chains by SEC experiments. It appears clear from our data that the linker chains have a very high affinity for some globin chains, especially the globin trimers *abc*. On the other hand, the monomer *d* (VI) fraction is indeed quite pure, while the fraction rich in trimer (IV) is contaminated by the presence of linker chains and a small amount of monomers. Although, the remaining fractions present a mixture of subunits, a detailed characterization was made for these subunits. Our new MALDI-TOF-MS analysis performed in this work showed that the monomer *c* presents four isoforms and the trimer, *abc*, is also characterized by two isoforms, T_1 and T_2. In addition, the contribution of the dimer of monomers was observed for two globin chains, *d* and *c*, and the presence of the tetramer, *abcd*, with *MM* at 68,400 kDa was detected. Finally, HbGp hemoglobin, at different iron oxidation states, is very similar regarding its subunits composition. Our present studies are consistent with literature reports on several other extracellular hemoglobins, such as the HbLt and HbAm. We believe our results represent a nice contribution and an important and necessary step in the complete characterization of HbGp oligomeric structure.

Acknowledgments

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