2008-07

Ferric species of the giant extracellular hemoglobin of Glossoscolex paulistus as function of pH: an EPR study on the irreversibility of the heme transitions

Comparative Biochemistry and Physiology B, New York, v. 150, n. 3, p. 292-300, 2008
http://www.producao.usp.br/handle/BDPI/49245

Comparative Biochemistry and Physiology, Part B 150 (2008) 292–300

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Ferric species of the giant extracellular hemoglobin of *Glossoscolex paulistus* as function of pH: An EPR study on the irreversibility of the heme transitions

Leonardo Marmo Moreira [a,b,*], Alessandra Lima Poli [a], Juliana Pereira Lyon [b], Jamil Saade [b], Antonio José Costa-Filho [c], Hidetake Imasato [a]

[a] Instituto de Química de São Carlos, Universidade de São Paulo, 13560-970, São Carlos, SP, Brazil
[b] Instituto de Pesquisa e Desenvolvimento, Universidade do Vale do Paraíba, 12244-000, São José dos Campos, SP, Brazil
[c] Instituto de Física de São Carlos, Universidade de São Paulo, 13560-950, São Carlos, SP, Brazil

ARTICLE INFO

Article history:
Received 10 February 2008
Received in revised form 19 March 2008
Accepted 21 March 2008
Available online 8 April 2008

Keywords:
Extracellular hemoglobin
Glossoscolex paulistus
Electron paramagnetic resonance (EPR)
Ferric heme
Hemichrome
Aquomet species
Transition irreversibility
Denaturation

ABSTRACT

The present article is focused on the transitions of ferric heme species of the giant extracellular hemoglobin of *Glossoscolex paulistus* (HbGp) induced by successive alterations in pH, involving alkaline and acid mediums. Electron paramagnetic resonance (EPR) is the spectroscopy used to evaluate the transitions that occur in the first coordination sphere of ferric ion as a consequence of ligand changes in a wide range of pH, since this tool is very sensitive to slight changes that occur in the heme pocket of paramagnetic species. This approach is adequate to obtain information regarding the reversibility/irreversibility that involves the heme transitions induced by pH, since the degree of reversibility is associated to the intensity of the changes that occur in the spatial configuration of the polypeptide chains, which is clearly associated to the first coordination sphere. The results demonstrate a significant degree of irreversibility of heme transitions, since the final species, which do not present any change after 6 h of its respective formations, are quite different of the initial species. The results denote that the more stable species are the bis-histidine (hemichrome) and pentacoordinate species, due to the properties of their ligands and to the mechanical influence of the respective subunits. EPR spectra allow to distinguish the types of hemichrome species, depending on the reciprocal orientation between the histidine axial ligands, in agreement with Walker's Classification [Walker, F.A., 1999. Magnetic spectroscopic (EPR, ESEEM, Mössbauer, MCD and NMR) studies of low-spin ferriheme centers and their corresponding heme proteins. Coord. Chem. Rev. 185–186, 471–534]. However, these transitions are not completed, i.e., the appearance of a determined species does not mean the total consumption of its precursor species, implying the coexistence of several types of species, depending on pH. Furthermore, it is possible to conclude that a "pure" EPR spectrum of aquomet ferric species is an important indicator of a high level of conservation referent to the "native" configuration of whole hemoglobin, which is only encountered at pH 7.0. The results allow to infer important physico-chemical properties as well as to evaluate aspects of the structure–activity relationship of this hemoprotein, furnishing information with respect to the denaturation mechanism induced by drastic changes in pH. These data are very useful since HbGp has been proposed as prototype of substitute of blood, thus requiring wide knowledge about its structural and chemical properties.

1. Introduction

Globins are heme-containing proteins, widespread in the three major kingdoms of life, that reversibly bind oxygen and other gaseous ligands. Despite the great diversity of their amino acid sequences, the basic functional unit is assumed to be a monomeric globin with a specific and highly conserved fold referred as the "globin-fold. Three types of globin have been described in annelids: (i) non-circulating intracellular globin; (ii) circulating intracellular globin; (iii) extracellular globin dissolved in circulating fluids (Bailly et al., 2007).

Considering the extracellular globins, it is important to notice that the symmetrical arrangements of multiple subunits are observed in invertebrate giant extracellular respiratory proteins. These include the cooper-containing hemocyanins, from arthropods, mollusks, and heme-containing respiratory proteins, such as those found in annelid worms. The most prevalent of these annelid complexes is known as either erythrocruorins or hexagonal bilayer hemoglobins (HBL) (Royer et al., 2006). Indeed, among the four types of existing respiratory proteins (hemocyanins, hemerytrins, chlorocruorins and hemoglobins) hemoglobins are the more widely distributed in vertebrate and invertebrate animals (Arndt and Santoro, 1998).

* Corresponding author. Instituto de Pesquisa e Desenvolvimento da Universidade do Vale do Paraíba, São José dos Campos-SP, Av. Shishima Hifumi, 2911 Zip Code: 12244-000, Bairro Urbanova, São José dos Campos, SP, Brazil. Tel.: +55 12 3947 1124.
E-mail address: leonardomarmo@univap.br (L.M. Moreira).

1096-4959/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2008.03.020

The giant extracellular hemoglobins represent the summit of complexity of the heme proteins that carries oxygen (Vinogradov, 2004). This is due to their extraordinary supramolecular masses, approximately 3.6 MDa; high cooperativity; redox and oligomeric stabilities, etc. These interesting characteristics are associated to the existence of a central substructure that maintains the total protein assembly (Ohtsuki and Crewe, 1983), which is dependent on the type of erythrocruorin, in agreement with the classification of Royer et al. (2007). These relevant properties provoked the interest of various research groups in their respective studies in order to improve the knowledge of the structure–function relationship of hemoproteins. The oxygen binding properties of the giant extracellular hemoglobin of *Oligobrachia mashikoi*, for instance, are regulated by divalent cations, which preferentially bind to the oxy-form (Aki et al., 2007). Significant effort to employ this class of hemoglobin as blood substitute, i.e., artificial oxygen carrier has been developed (Harrington et al., 2007). Indeed, the high autoxidation resistance, which is found in the giant extracellular hemoglobins, is an important prerequisite to this kind of physiological application (Harrington et al., 2007; Rousselot et al., 2006; Hirsch et al., 1997). Although non-vertebrates are phylogenetically more primitive than vertebrates, the high variability encountered in their hemoglobins reflects specialization and adaptation to a greater range of operating conditions than those found in vertebrates (Weber and Vinogradov, 2001). However, compared with vertebrate hemoglobins, much less is known about the relations between their physiological functions and their molecular structures at the atomic level (Weber and Vinogradov, 2001).

The hemoglobin of the annelid *Lumbricus terrestris* (HbLt) is the most studied erytrocruorin. Indeed, HbLt was the first protein reported to be crystallized, in 1840 by Hünefeld, one of the first proteins investigated in Svedberg's initial ultracentrifugation experiments and an early molecular subject of electron microscopy (Royer et al., 2006). Quaternary structure of this protein presents approximately 180 polypeptide chains, involving 144 globin subunits and 36 non-globin chains. The heme group, which is responsible for the biological transport of molecular oxygen, is present only in the globins, while the non-globin subunits, called *Linkers* chains, present only structural function. The oligomeric assembly is organized as two superposed hexagonal discs, being known as "bracelet model". This supramolecular arrangement presents the *Linker* chains in a central position and the globins in a more peripheral disposition. In this way, the 144 globin subunits are more directly related to the hexagonal vertices of the "bracelet". Thus, each "bracelet" has twelve dodecamers, i.e., each hexagonal vertex is associated to one dodecamer, being that each dodecamer is constituted for 3 tetramers *abcd*. In this time, each *abcd* tetramer is constituted by one *abc* trimer, which presents its subunits connected by disulfide bounds, and one *d* monomer that is maintained in the tetramer by weak interactions. On the other hand, the *Linker* subunits that occur mainly in a central position of the "bracelet" present four kind of chains (L1, L2, L3, L4), in which sequences of amino acid residues have been recently determined (Kao et al., 2006).

Therefore, the dodecamer is a fraction (1/12) of the whole protein, being associated then to $(abc)_3d_3L_3$, where L stands for the *Linker* chains. Vidugiris et al. (1993) argue that the retention of only partial cooperativity by 1/12 arrangement implies that full cooperativity is dependent on the presence of a complete hexagonal bilayer structure. On the other hand, Fushitani and Riggs (1991) suggest that 1/12 arrangement would have a fundamental role in the cooperative mechanism as primary functional unit. This controversial topic illustrates the complexity of the structure–function relationship of this class of hemoproteins, denoting that the influence of this complex structure in the cooperative behavior of the giant extracellular hemoglobins is still a great challenge. In fact, the cooperativity originated from different mechanisms in *L. terrestris* hemoglobin compared to vertebrate hemoglobins (Vidugiris et al., 1993). Strand et al. (2004) described the subunit contacts in the dodecamer of HbLt at 2.6 Å resolution. These authors argue that the packing of residues in the neighborhood of the distal ligand bound to the metallic center corresponds to a rather crowded distal pocket, allowing to infer a representative impact in the hemoglobin oxygen affinity by the concomitant effects of stabilizing the bound ligand and restricting ligand access to the ferrous center. Furthermore, they identified in the chains *a*, *b*, and *d* a large side chain from aromatic residues at B10, where the chains *b* and *d* present a residue of tryptophan and the subunit *a* has a residue of phenylalanine, while the *c* chain presents a residue of leucine, which is associated to a less intense steric hindrance. This fact could be associated to the well-known resistance to autoxidation found in the giant extracellular hemoglobins, including that from *G. paulistus*, which is evaluated in the present work.

The hemoglobin of *Glossoscolex paulistus* (HbGp) belongs to the same class of the HbLt and exhibits similar structural characteristics to this hemoglobin (Oliveira et al., 2007, 2008). *G. paulistus* is an annelid found in Araras, Rio Claro and Piracicaba, which are cities of the São Paulo state in Brazil (Righi, 1972). It is important to notice that the conditions of the protein medium represent a decisive factor to determine the oligomeric conformations. pH changes, for example, can alter drastically the spatial configurations of the polypeptide chains as well as the first coordination sphere of the heme metallic center. In fact, an intense alkaline dissociation is well-characterized at pH 9.0, whereas the native assembly of HbGp is well-conserved at pH 7.0 (Santiago et al., 2007; Moreira et al., 2008a; Gelamo et al., 2004; Bispo et al., 2005). Recent results have suggested that protons present a very important function in the assembly of the HbGp in its integral state (Bispo et al., 2005, 2007).

Fluorescence studies of HbGp have shown that the dissociation of its oxy-form at alkaline pH 9.0 is not complete, resulting in $(abcd)_2$ fraction as the main product, while the met-form shows complete dissociation into trimers, *Linkers* and monomers (Perussi et al., 1990; Agustinho et al., 1996). This difference between the iron oxidation states is an example of the oligomeric implications associated to the properties of the first sphere of coordination in giant hemoglobins. Alkaline oligomeric dissociation is related to a simultaneous autoxidation process as a function of the water solvent accessibility increase into the heme pocket, which, in its turn, favors even more the oligomeric dissociation, creating a dissociation–autoxidation–dissociation synergic process, since the ferric species presents lower structural stability than ferrous species (Zhu et al., 1996). In this way, a kind of dissociation–autoxidation cooperative effect occurs when medium perturbations originate an initial process of oligomeric dissociation. In acid medium, a similar process of oligomeric dissociation also takes place (Mainwaring et al., 1986). However, this disassembly process is much less intense than in alkaline medium, which must be associated to the acid isoelectric point (pI) of this class of hemoglobin (Vinogradov, 2004; Mainwaring et al., 1986). Mainwaring et. al. (1986) determined that the isoelectric point (pI) of HbLt is 5.5. Indeed, Arndt et al. (2003) argue that the acid pI of the extracellular hemoglobin of *Biomphalaria glabrata* (HbBg), which is 4.6, is responsible for the expansion of this protein at pH 7.5, when compared with pH 5.0, due to repulsion of negative charges in excess within the protein, changing its structure but preserving its absolute molecular mass. Thus, when this decrease of the compact character of subunits takes place, as a consequence of the anionic repulsion between the subunits, the intensity of the intra- and inter-chains contacts would be smaller, favoring a subsequent dissociation process. In this way, the acid pI of the giant extracellular hemoglobins seems to be an important factor to determine various properties of the structure–activity relationship, since it is associated to the intensity of cohesion of the oligomeric assembly and, consequently, is related to the level of hydrophobic isolation of the heme pocket of its chains.

In the present work, the possibility of reversion of the HbGp ferric heme transitions in a wide range of pH is analyzed, involving acid and alkaline mediums, in order to evaluate the influence of the polypeptide

chains on the heme transitions. This proposal can be an interesting contribution to improve our understanding upon the mechanisms of unfolding of hemoproteins as well as their influences on the reversibility/irreversibility of the ferric species of the heme pocket. This study is an original contribution, since, in our knowledge, in spite of the great relevance physico-chemical of this kind of evaluation, small data focused on reversibility studies *via* EPR can be obtained in the literature.

2. Materials and methods

2.1. Protein preparation

The giant extracellular hemoglobin of *G. paulistus* was prepared using freshly drawn blood from worms. The blood sample was purified by ultra centrifugation and gel filtration in Sephadex G-200 column at pH 7.0 (Imasato et al., 1995).

2.2. EPR measurements

X-band (9.5 GHz) EPR spectra were measured on a Brucker Elexsys E580 spectrometer at 4 and 12 K. The temperature was controlled by an Oxford ITC 503 cryogenic system. EPR samples (50 μL) containing approximately 20 mg mL^{-1} of the protein were frozen by immersion in liquid nitrogen and then placed in the spectrometer rectangular cavity. The microwave power was 4.0 mW and other acquisition conditions, such as modulation amplitude were adjusted to achieve optimal signal-to-noise ratio without signal distortion or saturation. All EPR data were corrected by subtracting a baseline corresponding to the EPR signal of the buffer. In order to evaluate a specific sequence of ferric heme transitions, the second ferric heme transition through pH changes was developed after 3 h from the first transition to each set of EPR measurements. Subsequently, after 6 h from the first measurement (3 h after the second measurement), a definitive spectral analysis was performed to evaluate if the final species undergone any spectral change.

3. Results

Fig. 1 demonstrates a typical axial spectrum correspondent to an aquomet ferric species with the couple of peaks with $g \sim 6.08$ and 2.01, which is encountered at pH 7.0. This species, which presents a water solvent molecule coordinating the ferric ion as sixth ligand of this metallic center, is practically "pure" in the respective spectrum, suggesting a great stability of this species at pH 7.0 as well as a highly conserved character to the protein assembly when compared with the native ferrous form of HbGp. Therefore, this spectrum is associated to a "native" conformation of the integral hemoglobin, i.e., the ferric species is highly conserved. However, this "native" conformation of whole hemoglobin of HbGp is very sensitive to any perturbation in the medium, especially pH changes. In this way, slight modifications in pH can generate drastic alterations in the first coordination sphere of the heme as a consequence of mechanical changes that occur in the polypeptide chains. This fact is evidenced by Fig. 2, which denotes an EPR spectrum quite distinct of that presented in Fig. 1. In fact, Fig. 2 also presents a spectrum obtained at pH 7.0, with the unique difference that this spectral measurement was obtained after a pH transition 7.0→10.5→7.0. Thus, this spectrum presented in Fig. 2 is a strong evidence of the significant irreversibility of the transitions involving ferric species of HbGp. The occurrence of a significant dissociative behavior involving the oligomers that constitutes HbGp at pH 9.0 is well established (Santiago et al., 2007; Moreira et al., 2008a; Gelamo et al., 2004; Bispo et al., 2005). In this way, pH 10.5 would be a condition that allows the oligomeric dissociation and makes the possibility of rearrangement of the protein assembly as a consequence of a significant loss of intra- and inter-chains contacts difficult.

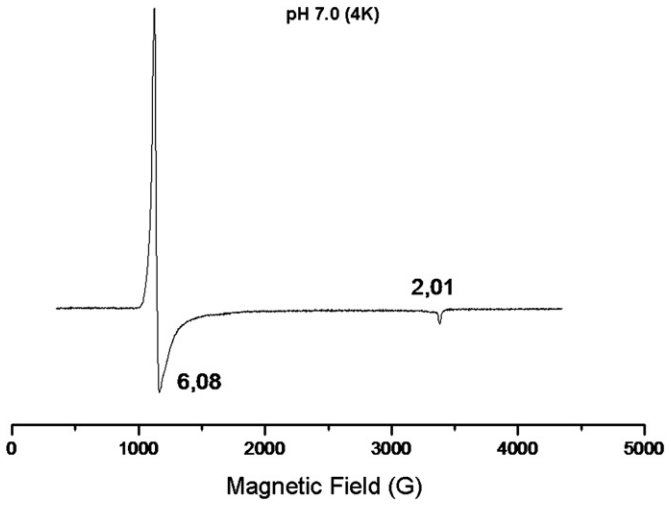

Fig. 1. Electron paramagnetic resonance spectrum of the giant extracellular hemoglobin of *G. paulistus* (met-HbGp) at pH 7.0 with the respective *g*-values indicated in each spectral peak. Conditions of the sample in the spectral measurement: 20 mM Tris–HCl buffer at pH 7.0 (T=4 K). EPR conditions: modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 4.0 mW; microwave frequency, 9.4784 GHz.

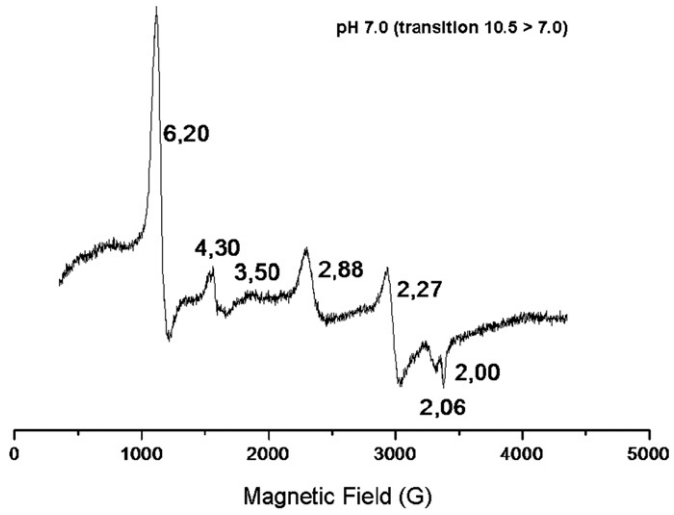

Fig. 2. Electron paramagnetic resonance spectrum of the giant extracellular hemoglobin of *G. paulistus* (met-HbGp) at pH 7.0, after pH transition 7.0→10.5→7.0, with the respective *g*-values indicated in each spectral peak. Experimental conditions are the same as forFig. 1.

Fig. 2 indicates the presence of significant coexistence of a great number of species. The spectral lines that present *g* values 2.27 and 2.88 indicate the presence of the called "hemichrome B", i.e., a "conventional" bis-histidine ferric heme complex with an usual spin state $(d_{xy})^2(d_{xz},d_{yz})^3$. This "hemichrome B" represents, according to the classification proposed by F. Ann Walker (Walker, 1999), the "Type II" complex, which is associated to an arrangement reciprocally parallel of the two histidine ligands, which are coordinated to the ferric ion in the axial sites. This disposition of axial ligands in the hemichrome B or Type II generates an electronic configuration considered "pure" low-spin state. This occurs due to the fact that this configuration presents a more intense ligand field as a consequence of a more pronounced superposition between molecular orbitals from ligands and metallic ion.

Fig. 2 also presents the spectral peak approximately $g \sim 3.50$, which corresponds to the so called "Type I" complex in accordance to Walker's Classification, indicating the hemichrome (bis-histidine complex) that presents a reciprocally orthogonal orientation between its axial histidine ligands (Walker, 1999; Walker, 2004; Moreira et al.,

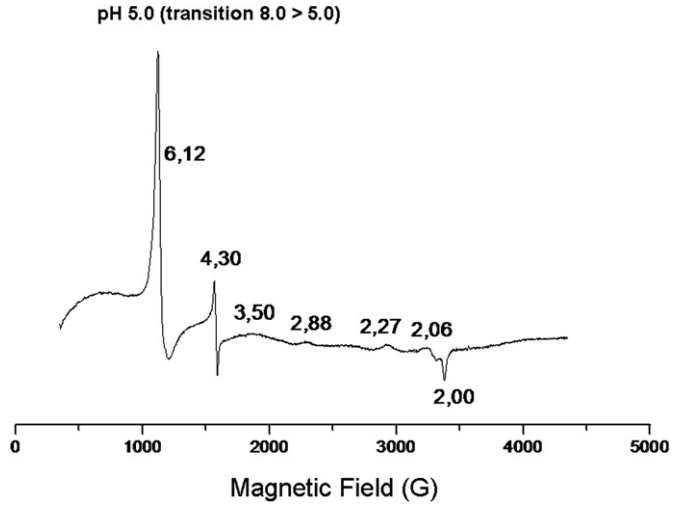

Fig. 3. Electron paramagnetic resonance spectrum of the giant extracellular hemoglobin of *G. paulistus* (met-HbGp) at pH 5.0, after pH transition 7.0→8.0→5.0, with the respective *g*-values indicated in each spectral peak. Experimental conditions are the same as for Fig. 1.

2004). This ferric heme species of "Type I" is also called "strong" g_{max} complex as a function of its great *g* value. This "Type I" hemichrome, which also shows a usual spin state $(d_{xy})^2(d_{xz},d_{yz})^3$, still presents the denomination *HALS* (Highly Anisotropic Low-Spin) species. It is worth to register that the distinction among these hemichromes is possible only through EPR spectroscopy, since the energy difference between these configurations is very small.

Indeed, Zaric et al. (2001) investigated the factors determining conformations of imidazole from histidine axially coordinated to heme in heme proteins by analyzing 693 hemes in 432 different crystal structures of heme proteins from Protein Data Bank (PDB). In this expressive collection, 65 were bis-histidine-ligated hemes and the energy balance between the two forms with usual spin state is the result of crystal field stabilization effects favoring the parallel form and steric effects that favor the perpendicular disposition. In this way, in heme model systems (metallic porphyrin complexes), the orientation of axial ligands can depend on crystal field stabilization effects or on steric effects caused by substituents on axial ligands on the porphyrin. On the other hand, in heme proteins, the heme does not possess bulky substituents, but the protein environment can have a steric influence on the orientation of the axial ligands. Medakovic and Zaric (2003) developed quantum chemical (DFT) calculations on heme model systems with non-substituted Fe-porphyrin core for the different orientation of the axially coordinated imidazoles. Their results indicate that perpendicular orientation (Type I hemichrome) can be explained by steric effects caused by propionic groups of porphyrin ring and by the histidine backbone. McGarvey (1998) has described a more detailed explanation for the occurrence of the *HALS* (Type I) hemichrome, considering that imidazole planes, which were initially in a parallel arrangement, adopt a perpendicular configuration, which is similar to a tetragonal distornal (Jahn–Teller effect). Therefore, the heme avoids a classic Jahn–Teller effect, with the axial ligands preferring the perpendicular orientation, which shows smaller orbitals overlap with the iron orbitals than the parallel orientation, causing a smaller splitting of iron *d* orbitals. In fact, Rieger (1994) has reported that Type I hemichrome presents an apparent near-degeneracy of the d_{xz} and d_{yz} orbitals that is consistent with the crystal structure, which showed that the two ligands lie in perpendicular planes because of the similar orbitals overlap to d_{xz} and d_{yz}.

It is important to notice that the Walker's Classification still presents the "Type III" species with an unusual spin state $(d_{xz},d_{yz})^4(d_{xy})^1$ (Walker, 1999), which would not be encountered in Fig. 2.

Fig. 2 shows a spectral line in $g \sim 4.3$, which is related to the non-heme ferric species and is probably associated to subunits that were profoundly affected by the protein unfolding, characterizing the occurrence of a pronounced denaturation process. The presence of the couple of peaks that characterizes the axial spectrum ($g \sim 6.20$ and 2.00) denotes that the several species formed do not represent the total consumption of the original aquomet species, which is a characteristic inherent to the equilibria involving ferric species of hemoglobin (Svistunenko et al., 2000). Furthermore, the slight asymmetry of the intense peak encountered in $g \sim 6.0$ is an evidence of an initial formation of pentacoordinate ferric heme species, in agreement with previous works (Moreira et al., 2006; Moreira et al., 2008b). This complex equilibrium is characterized by a significant presence of the species that denote more drastic protein unfolding, indicating that the stability and intensity of the presence of hemichromes and pentacoordinate species is deeply associated with the degree of spatial arrangement change of the quaternary architecture. It is important to notice that previous studies demonstrated that the native and reconstituted isolated *d* monomers of HbGp also present similar ferric heme transitions in acid and alkaline mediums, suggesting that the "globin-fold" presents a general characteristic of formation of hemichrome and pentacoordinate species, independently of the level of organization of the quaternary assembly (Ribelatto et al., 2005; Ribelatto et al., 2006).

Fig. 3 raises an interesting evaluation regarding the transition 7.0→8.0→5.0. The medium conditions inherent to pH 8.0 are insufficient to produce the protein dissociation that takes place at pH 9.0. Therefore, at pH 8.0 the degree of reversibility of the ferric species could be significant, since the medium conditions at pH 8.0 are not sufficient to produce the intense protein dissociation that occur at pH 9.0. Actually, when compared with Fig. 2 that corresponds to the pH transition 7.0→10.5→7.0, the pH transition 7.0→8.0→5.0 presented in Fig. 3 denotes the decisive character of the alkaline dissociation at pH 9.0 as a kind of "point of inflection", which intensifies the irreversibility of the transitions as a consequence of the disorganization obtained with the intense separation between subunits and oligomeric fractions.

Fig. 4 presents the spectrum obtained of the final protein configuration associated to the transition 7.0→6.0→7.0. Therefore, this sample was adjusted to pH 7.0 after a slight transition until pH 6.0. In spite of the well well-known stability of the aquomet ferric species

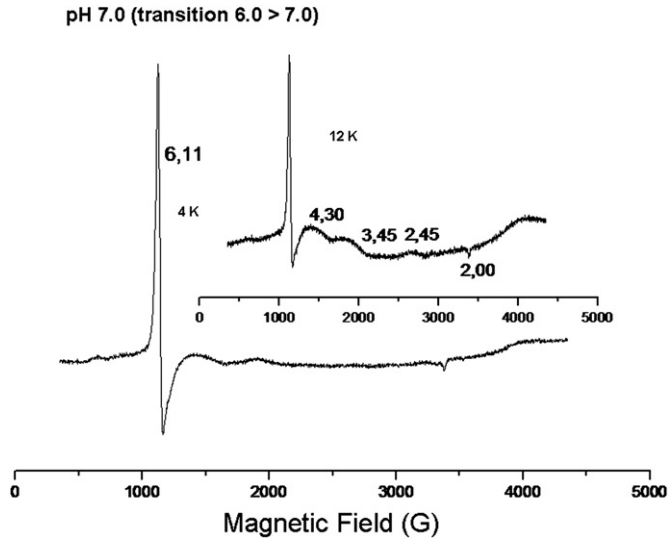

Fig. 4. Electron paramagnetic resonance spectrum of the giant extracellular hemoglobin of *G. paulistus* (met-HbGp) at pH 7.0, after pH transition 7.0→6.0→7.0, with the respective *g*-values indicated in each spectral peak. Experimental conditions are the same as for Fig. 1.

in slightly acidic medium (Moreira et al., 2006; Moreira et al., 2008b), it is possible to detect a significant change of this spectral profile when compared with the original spectrum of the "native" aquomet species obtained at pH 7.0 (Fig. 1). However, when compared with the spectrum presented in Fig. 2, it is evident that this heme transition is much less intense, illustrating, one more time, the great factor of oligomeric disorganization generated by the intense alkaline dissociation at pH 9.0.

Fig. 5 represents the spectrum generated by the transition 7.0→5.0→7.0. In spite of the evident spectral difference as compared with the "native" aquomet species spectrum, which was obtained without any pH transition (Fig. 1), in this transition 7.0→5.0→7.0 a drastic spectral alteration is not observed, similarly to the spectral behavior showed by the transition 7.0→6.0→7.0 (Fig. 4). Probably, the acid pI of HbGp is associated to this process. Indeed, only with a predominance of positive charges in the hemoglobin, as a function of a pH value significantly below pI, an acid dissociation would occur, favoring an irreversible separation between the subunits due to the natural difficulty of reorganization of the chains when pH returns to neutrality. In this case, this reassembly would be precluded as a consequence of the loss of inter- and intra-subunits contacts.

Fig. 6 presents several EPR spectra that were obtained in the acid range of pH through a conventional way, i.e., the transitions were developed in a direct form, such as 7.0→6.0, 7.0→5.0 etc. This figure is very representative due to the evident transition that occurs below pH 4.0. In fact, it is possible to observe a more pronounced spectral change between the spectra obtained in pH values 4.0 and 3.5. In previous article a similar transition in this pH range was also detected by UV–vis (Moreira et al., 2006). In pH values below pH 3.5, the transitions become more drastic, with more intense peaks. Thus, this more drastic transition must be associated to the well established acid pI (isoelectric point) of the giant extracellular hemoglobins. In this way, it is possible to propose that below pI, the predominance of cationic sites affects the spatial conformation of the polypeptide chains in a decisive way.

Fig. 7 represents several pH transitions, involving slight changes as well as drastic pH alterations. The transitions 7.0→6.0→7.0, 7.0→5.0→7.0, and even the more radical change 7.0→4.0→8.0, do not modify the original spectrum obtained at pH 7.0 in a significant way. In this way, we can propose the existence of two decisive points that determine the degree of transition between ferric heme species

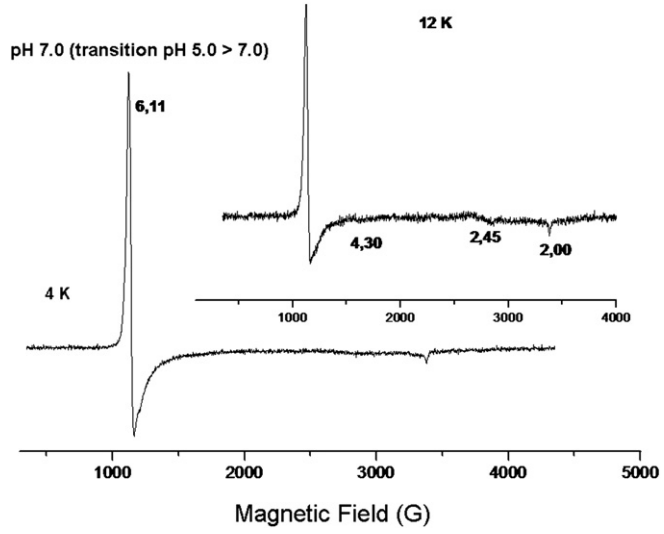

Fig. 5. Electron paramagnetic resonance spectrum of the giant extracellular hemoglobin of *G. paulistus* (met-HbGp) at pH 7.0, after pH transition 7.0→5.0→7.0, with the respective *g*-values indicated in each spectral peak. Experimental conditions are the same as for Fig. 1.

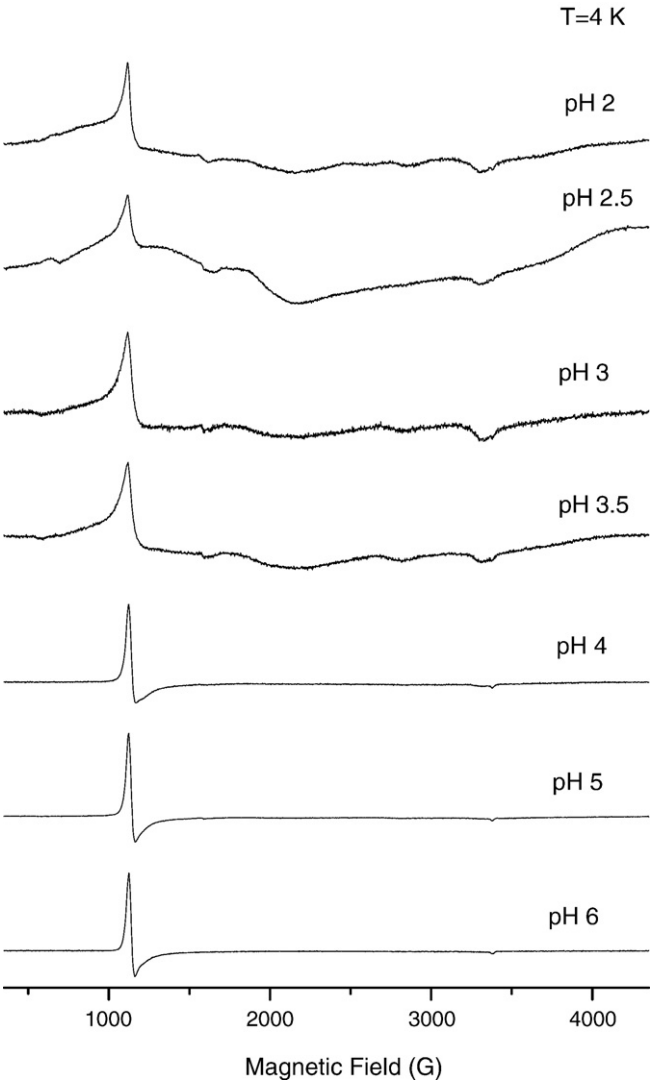

Fig. 6. Electron paramagnetic resonance spectra of the giant extracellular hemoglobin of *G. paulistus* (met-HbGp) at several pH acid values (pH values 6.0, 5.0, 4.0, 3.5, 3.0, 2.5 and 2.0) obtained through a simple transition from pH 7.0. Experimental conditions are the same as for Fig. 1.

induced by pH. Thus, initiating the analysis from the neutrality, a predominantly irreversible process occurs when the pH values 9.0 and 3.5 are exceeded. In this way, in these three transitions 7.0→6.0→7.0, 7.0→5.0→7.0, 7.0→4.0→8.0, the fluctuation between pH values does not reach the more decisive pH values, 9.0 and 3.5, regarding the structural stability of the native spatial conformation. These values represent drastic changes to HbGp that alter substantially its polypeptide arrangement around the heme pocket, becoming the changes in the first coordination sphere a definitive unfolding process, and affecting, for a consequence, the ligands coordinated to the ferric ion. The capital point of the drastic transition in alkaline medium that occurs at pH 9.0 is caused by a very intense oligomeric dissociation, in agreement with previous articles (Santiago et al., 2007; Moreira et al., 2008a; Gelamo et al., 2004; Bispo et al., 2005). On the other hand, in acid medium, the pI seems to be a decisive factor, since in pH values below pI, the predominance of anionic sites must affect significantly the intra- and inter-contacts between the polypeptide subunits, promoting definitive modifications in the heme pocket. In fact, previous works focused on HbLt have demonstrated significant oligomeric dissociation in pH values that are proximal to the pI of this giant extracellular hemoglobin.

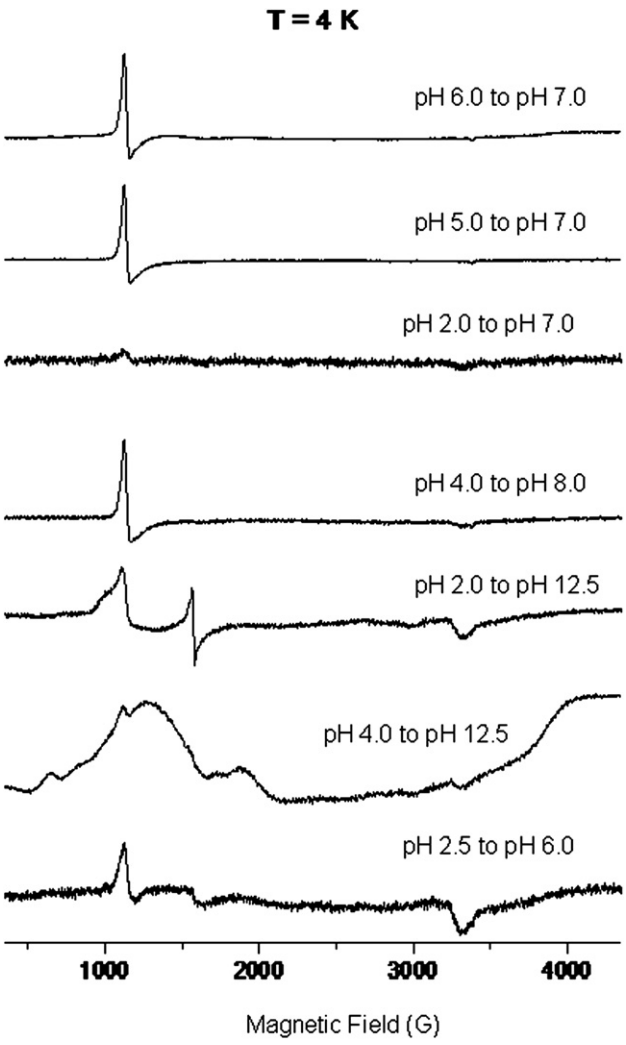

Fig. 7. Electron paramagnetic resonance spectra of the giant extracellular hemoglobin of *G. paulistus* (met-HbGp) at several pH values after drastic process of pH transition with pH acid values as intermediate steps (7.0→6.0→7.0; 7.0→5.0→7.0; 7.0→2.0→7.0; 7.0→4.0→8.0; 7.0→2.0→12.5; 7.0→4.0→12.5; 7.0→2.5→6.0). Experimental conditions are the same as for Fig. 1.

Fig. 7 also demonstrates the spectra referent to more drastic pH transitions, which are not inside of the limit range found between pH values 3.5 and 9.0, where the modifications developed in the heme pocket, as a consequence of the protein assembly unfolding, are less pronounced. Indeed, the transitions 7.0→3.0→7.0, 7.0→2.0→12.5, 7.0→4.0→12.5, 7.0→3.5→6.0, constitute drastic process of loss of the initial native configuration, being characterized by the coexistence between several ferric heme species. It is clear that the pH transitions 7.0→2.0→12.5 and 7.0→4.0→12.5 present the more drastic and heterogeneous spectral results, denoting a very complex coexistence between different species. Interestingly, these two spectra are significantly different from each other, indicating that the mechanism of protein unfolding is substantially distinct and profoundly dependent on the intermediate step.

4. Discussion

The present article demonstrates that, at least partially, the ferric heme transitions are irreversible in the giant extracellular hemoglobin of *G. paulistus* (HbGp) in acid and alkaline mediums. This work is in agreement with recent article focused on dynamic light scattering of the ferrous form of HbGp (Oxi-HbGp) as a function of pH and temperature, which demonstrated a very typical dissociative kinetics in alkaline medium, denoting that the metallic center oxidation is a kind of pre-requisite to a complex process of dissociation (Santiago et al., 2008). In fact, previous kinetic studies demonstrated that the Oxi-HbGp is very sensitive to perturbations in the external medium, such as pH, presence of surfactant or presence of exogenous ligands as, for example, cyanide (Poli et al., 2005, 2006). In this way, a synergic process occurs in the sequence autoxidation–dissociation–autoxidation, since the dissociation, which is the second step of this mechanism, generates a more accentuated autoxidation process as a function of the increase in the water solvent accessibility.

In present work, the level of cohesion in the whole hemoglobin is probably less effective when compared with the ferrous whole hemoglobin. Consequently, ferric whole hemoglobin (met-HbGp) must present a higher tendency to dissociation, in accordance with previous works involving acid and alkaline mediums (Perussi et al., 1990; Agustinho et al., 1996). In this context, it is important to notice the very interesting article of Arndt et al. (2003), that discuss the correlation between pI, protein cohesion and oligomeric stability. Indeed, this lower cohesion of the protein assembly allows a more significant permeability of the water solvent into the heme pocket, which presents, a priori, a highly hydrophobic character, decreasing the dielectric constant of the region around the heme, favoring significantly several transitions in the first coordination sphere.

In this protein with great mass, the mechanical influence of the polypeptide chains seems to be decisive to determine the ligands that

can coordinate the metallic center, independently of other factors. Actually, the spatial configuration of the 144 globin subunits is determinant to the configuration of the heme pocket, since several residues of amino acids are potential ligands that cannot coordinate the metallic center due to steric hindrance in the native condition. This occurs as a consequence of the highly conserved spatial disposition of the residues, which precludes that the residues can compete freely for the ferric ion, which is observed in ferric–porphyrin model complexes. It is important to register that the water solvent accessibility to the heme pocket, which is a decisive influence in the ligand changes, is clearly dependent on the spatial disposition of the polypeptide chains, i.e., the intensity of water accessibility into the heme pocket is a consequence of the degree of protein unfolding.

pH 7.0 represents a condition where HbGp shows a highly conserved quaternary structure with respect to the "native" state. In this way, the EPR spectrum obtained in these conditions is practically "pure", i.e., denotes the presence of only one species. However, initiating pH changes, the protein changes acquire irreversible character due to the higher affinity of the histidine to the ferric ion, when compared with the water ligand, which is the unique sixth ligand at pH 7.0. This occurs because the lateral chain of histidine is an imidazole group, which is a stronger field ligand to ferric ion (Fe(III)) than water molecule. Thus, the distal histidine easily displaces the water ligand of the sixth site of the metallic center. Consequently, the return to the initial configuration is much more difficult, i.e., it is a non-spontaneous process, since the stability of the imidazole-ferric ion ligation is significantly superior to the water–ferric ion ligation. Furthermore, other important factor must be considered in order to explain the irreversibility of these heme ferric transitions. This fact is the acquisition of more freedom of movements of each globin, allowing that each subunit can coordinate the metallic center without mechanical restrictions generated by the native protein assembly. This higher facility of movements is caused by the loss of inter- and intra-chains contacts due to the unfolding generated by pH changes, since these medium modifications affect the ionic contacts, as well as the hydrophobic interactions.

It is well established that the binding kinetics of non-vertebrate hemoglobins is strongly influenced by the structure of the heme cavity, particularly the size and polarity of residues occupying the distal portion that exert steric and dielectric effects (Weber and Vinogradov, 2001). The comparison of different hemoglobins suggests that the protein moieties can alter oxygen affinity using three broad mechanisms. Stereochemical differences in the proximal pocket can impact the reactivity of the heme iron or can increase affinity by providing favorable electrostatic interactions for a bound oxygen molecule. All of these three mechanisms have been found to contribute to the modulation of oxygen affinity in allosteric hemoglobins in their native states (Royer et al., 2005).

Besides the oxidation process, the increase in water permeability into the heme pocket originates a very interesting and complex transition between different species through the change in the ligands coordinated to metallic center. This mechanism is provoked initially by the breakage of the hydrogen bond between the distal histidine and the sixth ligand of iron. In the case of the ferrous species the sixth ligand is the oxygen molecule and to ferric species, reported in the present article, this ligand is a water molecule.

The highly hydrophobic character of the heme pocket in the hemeproteins, especially the erytrocruorins, is a decisive factor to stabilize the water molecule as sixth ligand of the metallic center when the oxidation takes place at pH 7.0. This occurs because the hydrophobic environment is not attractive to interact with a polar molecule such as H_20 and, consequently, when this ligand enter in the heme pocket, it will be immediately attracted by the cationic coordination center, which is the ferric ion (Fe(III)). Consequently, a new coordination occurs and the water becomes the sixth ligand of the first coordination sphere of the iron. Further of the heme pocket hydrophobicity, which precludes that the water interacts with other atoms of the heme pocket, the intense hydrogen bond with the distal histidine (E7) is also responsible for the stability of this water–ferric ion coordination. In fact, the water would be fixed in the distal position for two reasons: the ferric coordination and the hydrogen bond with the distal histidine.

In this way, without the addition of exogenous ligands, the displacement of the water from the sixth coordination site of the ferric ion would only occur when the dielectric constant of the heme pocket was altered and/or the distal histidine would become less fixed to allow its movement towards the metallic center. Indeed, the dielectric constant increase as a function of the higher permeability of the aqueous solvent into the heme pocket must destabilize the hydrogen bond between the sixth ligand (water) and the distal histidine, favoring the exit of this ligand from the first coordination sphere of the iron. On the other hand, the breakage of inter- and intra-subunits contacts turns the distal histidine free to develop a movement in the heme direction and to compete with water for the coordination of the ferric ion. Since histidine is a stronger ligand than water molecule to coordinate the ferric ion, this facility of movement is decisive to the distal histidine to dislocate the water ligand from the metallic center.

Thus, any medium perturbation that provides an oligomeric change is an agent that can provoke some ligand exchange at first coordination sphere of the ferric ion due to the structural inter-relationship between polypeptide chains and heme pocket. Indeed, the process of oligomeric dissociation, unfolding and denaturation that are observed in hemoglobins can be brought about by (i) addition of organic solvents, (ii) addition of denaturants, such as surfactants, (iii) heating, (iv) applying high pressures, and (v) changing the pH of medium (Venkatesh et al., 2003). Usually, these medium perturbations affect the protein assembly and promote protein unfolding, through the breakage of inter- and intra-subunits contacts. So, each polypeptide chain becomes less crowded and freer, in its molecular dynamics, to originate movements independently of the other subunits. Consequently, the distal histidine is free to coordinate the metal. Furthermore, in a kind of synergic mechanism, this unfolding process usually promotes a polarity increase into the heme pocket that destabilizes the water coordination, favoring more intensely the ligand exchange.

The posterior modifications between the types of hemichrome in more drastic medium conditions and even the formation of pentacoordinate species are consequences of this first process of decrease of cohesion of the protein assembly, which favors a continuum mechanism of subunit dissociation.

It is important to notice that previous works have demonstrated the occurrence of a process, at least partially irreversible, of oligomeric dissociation after pH changes (Moreira et al., 2008a; Santiago et al., 2008, 2007; Ribelatto et al., 2006, 2005; Agustinho et al., 1996, 1997). Likewise, it has been demonstrated that in alkaline (Moreira et al., 2008b) as well as in acid medium (Moreira et al., 2006), the heme ferric transitions caused by more drastic pH transitions propitiate a process of secondary structure loss, which is detected by Circular Dichroism (CD), that is intrinsically related to the modifications that occur in the first sphere coordination of the ferric center.

This approach can be an interesting tool in order to improve our understanding about the structure–function relationship of hemoproteins. Actually, an analysis based on Walker's Classification (Walker, 1999; Walker 2004) could furnish more detailed information regarding the occurrence of hemichromes in human hemoglobins, which are associated to the appearance of "Heinz Bodies" and hematological diseases (Rachmilewitz et al., 1969). In fact, few data are found in the literature with respect to the different species of hemichrome in human hemoglobins, which could improve the understanding of several relevant pathological processes of the vertebrates. The correlations between the occurrence of hemichromes

and the formation of channels of access to the solvent in vertebrate hemoglobins are well established (Robinson et al., 2003; Pesce et al., 2004). Furthermore, this mechanism has been associated to higher processes of denaturation and hemolytic anemia, which is characterized by the hemoglobin precipitation in the hemichrome form that constitutes the so called "Heinz Bodies" (Rachmilewitz et al., 1969; Robinson et al., 2003; Pesce et al., 2004).

5. Conclusions

The present article demonstrated that the ferric heme transitions of HbGp are predominantly of irreversible character. In fact, in all ranges of pH evaluated in the present work, it is evident that the pH transition in return to the original pH does not generate the initial configuration of the ferric hemes. Therefore, the pH transition promotes significant alteration in the heme pocket as well as in polypeptide chains, which inhibit the rearrangement of the initial configuration in the same original pH. This occurs as a consequence of steric hindrances and mechanic alterations of the subunit assembly as well as coordination of some endogenous strong field ligands, which preclude the obtaining of the initial organization. The intense alkaline dissociation and the acid pI determine decisive points of inflection that, when exceeded, accentuate the irreversibility of the ferric heme transitions.

Acknowledgements

The authors are also grateful to CNPq for doctoral and Post-doctoral fellowships to Leonardo Marmo Moreira. AJCF would like to thank CNPq and PRONEX/FAPESP/CNPq (Grant No. 03/09859-2).

References

Aki, Y., Nakagawa, T., Nagai, M., Sasayama, Y., Fukumori, Y., Imai, K., 2007. Oxygenation properties of extracellular giant hemoglobin from *Oligobrachia mashikoi*. Biochem. Biophys. Res. Commun. 360, 673–678.

Arndt, M.H.L., Santoro, M.M., 1998. Structure of the extracellular hemoglobin of *Biomphalaria glabrata*. Comp. Biochem. Physiol. 119B, 667–675.

Arndt, M.H.L., Oliveira, C.L.P., Régis, W.C.B., Torriani, I.L., Santoro, M.M., 2003. Small angle X-ray scattering of the hemoglobin from *Biomphalaria glabrata*. Biopolymers 69, 470–479.

Agustinho, S.C.M., Tinto, M.H., Imasato, H., Tominaga, T.T., Perussi, J.R., Tabak, M., 1996. Spectroscopic studies of the met form of the extracellular hemoglobin from *Glossoscolex paulistus*. Biochim. Biophys. Acta 1298, 148–158.

Agustinho, S.C.M., Tinto, M.H., Perussi, J.R., Tabak, M., Imasato, H., 1997. Fluorescence studies of extracellular hemoglobin of *Glossoscolex paulistus* in met form obtained by sephadex gel filtration. Comp. Biochem. Physiol., B 118, 171–181.

Bailly, X., Chabasse, C., Hourdez, S., Dewilde, S., Martial, S., Moens, L., Zal, F., 2007. Globin gene family evolution and functional diversification in annelids. FEBS J. 274, 2641–2652.

Bispo, J.A.C., Landini, G.F., Santos, J.L.R., Norberto, D.R., Bonafe, C.F.S., 2005. Tendency for oxidation of annelid hemoglobin at alkaline pH and dissociated states probed by redox titration. Comp. Biochem. Physiol., B 141, 498–504.

Bispo, J.A.C., Santos, J.L.R., Landini, G.F., Goncalves, J.M., Bonafe, C.F.S., 2007. pH dependence of the dissociation of multimeric hemoglobin probed by high hydrostatic pressure. Biophys Chem. 125, 341–349.

Fushitani, K., Riggs, A.F., 1991. The extracellular hemoglobin earthworm, *Lumbricus terrestris*: oxygenation properties of isolated chains, trimers, and a reassociated product. J. Biol. Chem. 266, 10275–10281.

Gelamo, E.L., Itri, R., Tabak, M., 2004. Small angle X-ray scattering (SAXS) study of the extracellular hemoglobin of *Glossoscolex paulistus*. Effect of pH, iron oxidation state, and interaction with anionic SDS surfactant. J. Biol. Chem. 279, 33298–33305.

Harrington, J.P., Kobayashi, S., Dorman, S.C., Zito, S.L., Hirsch, R.E., 2007. Acellular invertebrate hemoglobins as model therapeutic oxygen carriers: unique redox potentials. Artif. Cells Blood Substit. Immobil. Biotechnol. 35, 53–67.

Hirsch, R.E., Jelicks, L.A., Wittenberg, B.A., Kaul, D.K., Shear, H.L., Harrington, J.P., 1997. A first evaluation of the natural high molecular weight polymeric *Lumbricus terretris* hemoglobin as an oxygen carrier. Artif. Cells Blood Substit. Immobil. Biotechnol. 25, 429–444.

Imasato, H., Tinto, M.H., Perussi, J.R., Tabak, M., 1995. Fluorescence studies of extracellular hemoglobin of *Glossoscolex paulistus* obtained by gel filtration. Comp. Biochem. Physiol., B 112, 217–226.

Kao, W., Qin, J., Fushitani, K., Smith, S.S., Gorr, T.A., Riggs, C.K., Knapp, J.E., Chait, B.T., Riggs, A.F., 2006. Linker chains of the gigantic hemoglobin of the earthworm *Lumbricus terrestris*: primary structures of linkers L2, L3, and L4 and analysis of the connectivity of the disulfide bonds in linker L1. Proteins: Struct. Funct. Bioinf. 63, 174–187.

Mainwaring, M.G., Lugo, S.D., Fingal, R.A., Kapp, O.H., Vinogradov, S.N., 1986. The dissociation of the extracellular hemoglobin of *Lumbricus terrestris* at acid pH and its reassociation at neutral pH — a new model of its quaternary structure. J. Biol. Chem. 261, 10899–10908.

McGarvey, B.R., 1998. Survey of ligand field parameters of strong field d5 complexes obtained from the g matrix. Coord. Chem. Rev. 170, 75–92.

Medakovic, V., Zaric, S.D., 2003. Theoretical study on orientations of axially coordinated imidazoles in model systems of cytochromes. Inorg. Chim. Acta 349, 1–5.

Moreira, L.M., Ribelatto, J.C., Imasato, H., 2004. Ruffled and planar conformations of the porphyrin ring in complexes in heme proteins: physical–chemistry properties and spectroscopic implications. Quim. Nova 27, 958–963.

Moreira, L.M., Poli, A.L., Costa-Filho, A.J., Imasato, H., 2006. Pentacoordinate and hexacoordinate ferric hemes in acid medium: EPR, Uv–vis and CD studies of the giant extracellular hemoglobin of *Glossoscolex paulistus*. Biophys. Chem. 124, 62–72.

Moreira, L.M., Santiago, P.S., Almeida, E.V., Tabak, M., 2008a. Interaction of giant extracellular *Glossoscolex paulistus* hemoglobin (HbGp) with zwitterionic surfactant *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (HPS): effects of oligomeric dissociation. Colloids Surf., B 61, 153–163.

Moreira, L.M., Poli, A.L., Costa-Filho, A.J., Imasato, H., 2008b. Ferric species equilibrium of the giant extracellular hemoglobin of *Glossoscolex paulistus* in alkaline medium: HALS hemichrome as a precursor of pentacoordinate species. Int. J. Biol. Macromol. 42, 103–110.

Ohtsuki, M., Crewe, A.V., 1983. Evidence for a central substructure in a *Lumbricus terrestris* hemoglobin obtained with STEM low-dose and digital processing techniques. J. Ultrastruct. Res. 83, 312–318.

Oliveira, M.S., Moreira, L.M., Tabak, M., 2007. Partial characterization of giant extracellular hemoglobin of *Glossoscolex paulistus*: a MALDI-TOF-MS study. Int. J. Biol. Macromol. 40, 429–436.

Oliveira, M.S., Moreira, L.M., Tabak, M., 2008. Interaction of giant extracellular *Glossoscolex paulistus* hemoglobin (HbGp) with ionic surfactants: a MALDI-TOF-MS study. Int. J. Biol. Macromol. 42, 111–119.

Perussi, J.R., Imasato, H., Tabak, M., Meirelles, N.C., 1990. Fluorescence study of the extracellular hemoglobin of *Glossoscolex paulistus*. Physiol. Chem. Phys. Med. NMR 22, 159–168.

Pesce, A., Dewilde, S., Nardini, M., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T., Bolognesi, M., 2004. The human brain hexcoordinated neuroglobin three-dimensional structure. Micron 35, 63–65.

Poli, A.L., Moreira, L.M., Hidalgo, A.A., Imasato, H., 2005. Autoxidation studies of extracellular hemoglobin of *Glossoscolex paulistus* at pH 9: cyanide and hydroxyl effect. Biophys. Chem. 114, 253–260.

Poli, A.L., Moreira, L.M., Tabak, M., Imasato, H., 2006. SDS (sodium dodecyl sulfate) effect on the autoxidation of the *Glossoscolex paulistus* giant extracellular hemoglobin: kinetic studies at pH 7.0 and 9.0. Colloids Surf., B 52, 96–104.

Rachmilewitz, E.A., Peisach, J., Bradley, T.B., Blumberg, W.E., 1969. Role of haemichromes in the formation of inclusion bodies in haemoglobin H disease. Nature 222, 248–250.

Ribelatto, J.C., Poli, A.L., Moreira, L.M., Imasato, H., 2005. Pentacoordinate and hexacoordinate ferric hemes from the native and reconstituted d monomers of *Glossoscolex paulistus* extracellular hemoglobin: spectroscopic studies in acid medium. Quim. Nova 28, 829–833.

Ribelatto, J.C., Poli, A.L., Moreira, L.M., Imasato, H., 2006. Spectroscopic study of the equilibrium between hexacoordinated and pentacoordinated species of the native and reconstituted d monomers of the *Glossoscolex paulistus* giant extracellular hemoglobin in alkaline medium. Quim. Nova 29, 666–673.

Rieger, P.H., 1994. Electron paramagnetic resonance studies of low-spin d5 transition metal complexes. Coord. Chem. Rev. 135/136, 203–286.

Righi, G., 1972. Topography of circulatory system of *Glossoscolex paulistus* Michaelsen 1926 (Oligochaeta, Glossoscolocidae). Monit. Zool. Ital. 6, 19–36.

Robinson, V.L., Smith, B.B., Arnone, A., 2003. A pH-dependent aquomet-to-hemichrome transition in crystalline horse methemoglobin. Biochemistry 42, 10113–10125.

Rousselot, M., Delpy, E., La Rochelle, C.D., Lagente, V., Pirow, R., Rees, J., Hagege, A., Le Guen, D., Hourdez, S., Zal, F., 2006. *Arenicola marina* extracellular hemoglobin: a new promising blood substitute. Biotechnol. J. 1, 333–345.

Royer Jr., W.E., Zhu, H., Gorr, T.A., Flores, J.F., Knapp, J.E., 2005. Allosteric hemoglobin assembly: diversity and similarity, J. Biol, Chem., 280, 27477–27480.

Royer Jr., W.E., Sharma, H., Strand, K., Knapp, J.E., Bhyravbhatla, B., 2006. *Lumbricus* erythrocruorin at 3.5 Å resolution: architecture of a megadalton respiratory complex. Structure 14, 1167–1177.

Royer Jr., W.E., Omartian, M.N., Knapp, J.E., 2007. Low resolution crystal structure of *Arenicola* erythrocruorin: influence of coiled coils on the architecture of a megadalton respiratory protein. J. Mol. Biol. 365, 226–236.

Santiago, P.S., Moreira, L.M., Almeida, E.V., Tabak, M., 2007. Giant extracellular *Glossoscolex paulistus* hemoglobin (HbGp) upon interaction with cethyltrimetyhlammonium chloride (CTAC) and sodium dodecyl sulphate (SDS) surfactants: dissociation of oligomeric structure and autoxidation. Biochim. Biophys. Acta 1770, 506–517.

Santiago, P.S., Moura, F., Moreira, L.M., Domingues, M.M., Santos, N.C., Tabak, M., 2008. Dynamic light scattering and optical absorption spectroscopy study of pH and temperature stabilities of the extracellular hemoglobin of *Glossoscolex paulistus*. Biophys. J. 94, 2228–2240.

Strand, K., Knapp, J.E., Bhyravbhatia, B., Royer Jr., W.E., 2004. Crystal structure of the hemoglobin dodecamer from *Lumbricus* erythrocruorin: allosteric core of giant annelid respiratory complexes. J. Mol. Biol. 344, 119–134.

Svistunenko, D.A., Sharpe, M.A., Nicholls, P., Wilson, M.T., Cooper, C.E., 2000. A new method for quantification of spin concentration by EPR spectroscopy: application to methemoglobin and metmyoglobin. J. Magn. Reson. 142, 266–275.

Venkatesh, B., Ramasmy, S., Asokan, R., Rifkind, J.M., Manoharan, P.T., 2003. pH influenced metal ion coordination changes in reconstituted hemoglobin. J. Porphyrins Phthalocyanines. 7, 637–644.

Vidugiris, G.J.A., Harrington, J.P., Friedman, J.M., Hirsch, R.E., 1993. Absence of ligand binding induced terciary tertiary changes in the multimeric earthworm *Lumbricus terrestris* hemoglobin. J. Biol. Chem. 268, 26190–26192.

Vinogradov, S.N., 2004. The stoichiometry of the four linker subunits of *Lumbricus terrestris*. Micron 35, 127–129.

Walker, F.A., 1999. Magnetic spectroscopic (EPR, ESEEM, Mössbauer, MCD and NMR) studies of low-spin ferriheme centers and their corresponding heme proteins. Coord. Chem. Rev. 185–186, 471–534.

Walker, F.A., 2004. Models of the Bis-Histidine-ligated electron-transferring cytochromes. Comparative geometric and electronic structure of low-spin ferro- and ferrihemes. Chem. Rev. 104, 589–615.

Weber, R.E., Vinogradov, S.N., 2001. Nonvertebrates hemoglobins: functions and molecular adaptations. Physiol. Rev. 81, 569–628.

Zaric, S.D., Popovic, D.M., Knapp, E., 2001. Factors determining the orientation of axially coordinated imidazoles in heme proteins. Biochemistry 40, 7914–7928.

Zhu, H., Ownby, D.W., Riggs, C.K., Nolasco, N.J., Stoops, N.J., Riggs, A.F., 1996. Assembly of the Gigantic Hemoglobin of the Earthworm *Lumbricus terrestris*. J. Biol. Chem. 271, 30007–30021.