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Ferric species equilibrium of the giant extracellular hemoglobin of *Glossoscolex paulistus* in alkaline medium: *HALS* hemichrome as a precursor of pentacoordinate species

Leonardo Marmo Moreira[a,b,*], Alessandra Lima Poli[a], 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*

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Abstract

The present work is focused on the complex ferric heme species equilibrium of the giant extracellular hemoglobin from *Glossoscolex paulistus* (HbGp) in alkaline medium. EPR, UV–vis and CD spectroscopies were used in order to characterize the ferric heme species formed as a consequence of the medium alkalization as well as the oligomeric changes occurring simultaneously with heme transitions. EPR experiments allowed us to characterize the different hemichrome species in equilibrium, illustrating the small difference in spin state of this species and the complexity of the equilibira involving hemoglobin ferric species. The results emphasize the importance of the alkaline oligomeric dissociation, which is decisive to promote the heme ferric species transition as function of the increase in water accessibility to the heme pocket. In fact, the oligomeric dissociation in alkaline medium is a consequence of the intense electrostatic repulsion between anionic charges on the protein surface, since the isoelectric point (p*I*) of this hemoglobin is acid. This explains the more drastic aquomet–hemichrome-pentacoordinate species transition in alkaline medium as compared with the acid medium. However, these heme species transitions are not completed, i.e., the appearance of new species does not mean the total consumption of the precursor species. This equilibrium complexity is associated to the effective influence of oligomeric arrangement of this whole hemoglobin, which present 144 molecular subunits. The acid p*I* is probably an important factor to the structure–activity relationship of the giant extracellular hemoglobins.

Keywords: Extracellular hemoglobin; pH; Aquomet; Pentacoordinate; HALS hemichrome

1. Introduction

Giant extracellular hemoglobins, also called erythrocruorins or hexagonal bilayer hemoglobins (HBL), are highly cooperative respiratory biopolymers with an extraordinary molecular mass around 3.6 MDa [1]. This class of hemoglobins is considered the summit of structural complexity in proteins that carry oxygen [2,3] and it has been studied as a new promising blood substitute [4,5]. Although nonvertebrates 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 in vertebrates [3]. However, compared with the vertebrate hemoglobins, much less is known about the relations between their physiological functions and their molecular structures at atomic level [3]. The understanding of the influence of the whole complex structure on the cooperative behavior of giant extracellular hemoglobins is still a great challenge. Indeed, the cooperativity of these hemoglobins is originated from different mechanisms as compared to vertebrate hemoglobins [6].

The pH effect on biological systems has been widely investigated using various models to improve the research regarding biochemical process. Hemoglobin denaturation processes, for example, can be brought about by (i) addition of organic sol-

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

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vents, (ii) addition of denaturants, (iii) heating, (iv) application of high pressures, (v) pH changes [7].

Studies focused on the complexity of the equilibrium between hemoprotein ferric species as function of pH have been developed, especially with hemoglobins [8,9]. These studies have shown that a similar behavior regarding the heme species has been observed for different hemoproteins when pH change occurs. This may be associated with important aspects of their structure–activity relationship. Indeed, the pH changes, which originate an intense dissociative process for giant extracellular hemoglobins, especially in alkaline medium, lead to hemoglobin conformational changes, including allosteric phenomena, implying that the study of pH influence is an important step for understanding normal protein folding and its function [10,11].

Furthermore, the correlation between the oligomeric assembly changes and the alterations that takes place in the first coordination sphere of iron is also an important point to the understanding of the structure–activity relationship of hemoproteins. Walker and co-workers have studied and classified model complexes and hemoproteins regarding the different types of reciprocal orientation among axial ligands and the spatial conformations of the porphyrin ring [12–14]. These possible arrangements are decisive to determine the spin state and redox properties of the metallic center in hemoproteins. In the classification proposed by Walker and co-workers [12–14], Type I complex shows an usual spin state $(d_{xy})^2(d_{xz},d_{yz})^3$ and a reciprocally orthogonal orientation of the heterocyclic planes of the axial ligands. Type II complex presents also spin state $(d_{xy})^2(d_{xz},d_{yz})^3$, but parallel orientation between the respective ligand planes. Type III complex has an unusual spin state $(d_{xz},d_{yz})^4(d_{xy})^1$, and independent orientations of the axial ligand planes. This classification is especially important to hemichrome species, since these bis-histidine species clearly present two ligands containing cyclic structures of the imidazole group. Walker has also discussed the significant occurrence of bis-histidine species in cytochromes and has compared their geometric and electronic structures [15]. Zaric et al. [16] have investigated the factors that determine conformations of the imidazole axially coordinated to heme in heme proteins by analyzing 693 hemes in 432 different crystal structures from the Protein Data Bank (PDB). In this expressive collection, 65 conformations were bis-histidine-ligated hemes, illustrating the considerable occurrence of this species in heme proteins.

In this context, the giant extracellular hemoglobin of *Glossoscolex paulistus* (HbGp) is an excellent model system to evaluate the complexity of the species equilibrium as function of pH, mainly in alkaline medium, due to the intense dissociation of its 180 structural subunits in this condition. HbGp is similar to other annelid hemoglobins, especially hemoglobin of *Lumbricus terrestris* (HbLt), which is one of the most extensively studied hemoglobins. HbLt was the first protein 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 [1]. HbLt consists of about 180 polypeptide chains in an arrangement of two heme-containing subunits (monomer M and disulfide-bonded trimer T) and non-globin linker subunits ("Linker" chains) with molecular weights in the range 24–32 kDa assembled as a "bracelet model" of quaternary structure. In a recent work, the amino acid sequences of the four kinds of "Linker" chains (L1, L2, L3 and L4) have been determined [17]. HbGp is also constituted by a large number of subunits containing heme groups with molecular weights in the range of 16–19 kDa forming a monomer of 16 kDa and a trimer of 52 kDa and non-heme structures with molecular weights in the range 24–32 kDa [18], conferring to the whole protein a minimum molecular weight of 3100 kDa and a double layered hexagonal oligomeric structure [19,20].

The intense alkaline dissociation of the oligomeric structure of HbGp, around pH 9.0 has been characterized [11,21] and its impact on the polypeptide arrangement is very significant, since the giant extracellular hemoglobin presents an extraordinary mass of 3.6 MDa and its 180 constituent subunits lose their packed assembly upon dissociation. The oligomeric dissociation favors the increase of the water solvent permeability into the heme pocket, originating typical changes in the first coordination sphere of the iron.

In the present work, transitions between HbGp ferric species in alkaline medium are evaluated as function of pH, using electronic optical absorption (UV–vis), electron paramagnetic resonance (EPR) and circular dichroism (CD) spectroscopic techniques. The results are compared with our previous work that focus on the ferric species of HbGp in acid medium [8] and also with several works from the literature. A pattern of ferric species transitions, which is discussed in details, seems to be shared by various hemeproteins.

2. Materials and methods

2.1. Protein preparation

Giant extracellular hemoglobin from *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 columm at pH 7.0 [20].

2.2. UV–vis measurements

Electronic absorption spectra in the UV–vis region were obtained in the wavelength range of 250–700 nm as a function of pH, using a SHIMADZU UV-1601 PC spectrophotometer at room temperature. The absorbance of the samples at 415 nm was kept below 1.0 using a 1 cm path length cell, with concentration of approximately 0.2 mg mL^{-1} [22].

2.3. 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.

2.4. CD measurements

Far-UV CD spectra (190–250 nm) were obtained in a JASCO J715 (Jasco Co., Japan) as a function of pH employing the 0.2 cm path length cylindrical cell. Protein concentration was 0.22 mg mL^{-1} based on $\varepsilon_{415} = 3.13$ mL mg^{-1} cm^{-1} [23].

3. Results

3.1. UV–vis measurements

Fig. 1 presents the UV–vis data for HbGp in alkaline medium. At pH 7.0, it is possible to observe a characteristic spectrum of an aquomet species with bands at 405 nm (Soret band), 501 nm (Q band) and 628 nm (ligand-to-metal charge transfer (LMCT) band). The alkalization promotes a pronounced transition starting at pH 8.0, where the spectrum is significantly modified and a typical hemichrome spectrum (ferric bis-histidine species), characterized by bands at 535 and 567 nm is observed (Fig. 1). Further alkalization gives rise to a new transition, initiating a spectrum assigned to a pentacoordinated heme ferric species. Such assignment is well established in the literature and is based on the Soret band blue-shift, along with the decrease of the intensity of this band, which are thus attributed to a hexacoordinate-to-pentacoordinate heme transition [24–50]. This heme ferric transitions characterization is corroborated by the appearance of a typical LMCT band (around 600–605 nm) of pentacoordinate species in alkaline medium (see inset of Fig. 1) [28,51,52].

The spectra indicate that, probably, two ferric pentacoordinate species are coexisting in equilibrium. The intense shoulder approximately at 363 nm, which is the spectral profile of the Soret band in the more drastic alkaline conditions evaluated in the present work, is very suggestive of a pentacoordinate species presenting a water molecule as fifth ligand [24,25,34–36]. This species is in equilibrium with the typical pentacoordinate species, which shows the Soret band at 396 nm, indicating that the fifth ligand, in this case, is a histidine residue most likely from proximal site. The presence of water as fifth ligand in alkaline medium seems to be less representative than in acid medium [8], suggesting that the basic conditions are less drastic in terms of conformational changes when compared with the acid environment, at least, regarding to the rupture of both iron-histidine ligations [34,35]. Besides, in acid medium, the heme propionates can be protonated avoiding the salt bridges with the basic amino acid residues, which implies that in acid medium the heme could be free to rotate around the αγ heme axis as observed with methylated propionates. Indeed, Santucci et al. [53] reported that propionates play an important role through the salt bridges, especially between the 6-propionate and CD3 residue of horse heart myoglobin. Also the re-orientation toward the "correct" orientation perturb only local peptide domain.

It is worth noting that those transitions between heme ferric species are not complete, i.e., the appearance of new species does not mean the total elimination of the previous species. In any case, a more drastic alkalization favors the formation of the final species, which is the pentacoordinate species. The intensification in the alkalization process favors the aquomet–hemichrome-pentacoordinate species formation sequence, in this order.

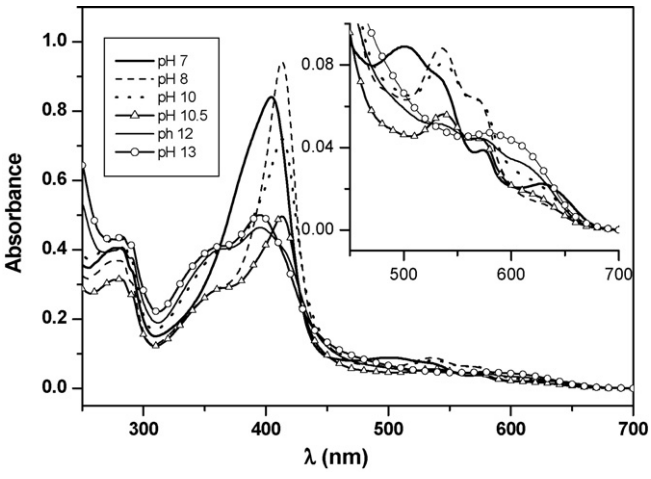

Fig. 1. Absorbance spectra of *Glossoscolex paulistus* hemoglobin in alkaline medium. *Conditions*: 20 mM Tris–HCl buffer; HbGp concentration was 0.2 mg mL^{-1}.

3.2. EPR measurements

Fig. 2 shows the EPR spectrum of HbGp at pH 7.0, which presents two resonances centered at ∼6.0 and ∼2.0. This is characteristic of a high-spin Fe(III) center, fingerprint of the aquomet species, in agreement with the UV–vis results. As pH is raised, extra signals are observed in the low spin region (Fig. 3). The *g*-values of these new peaks are typical of a "pure" low-

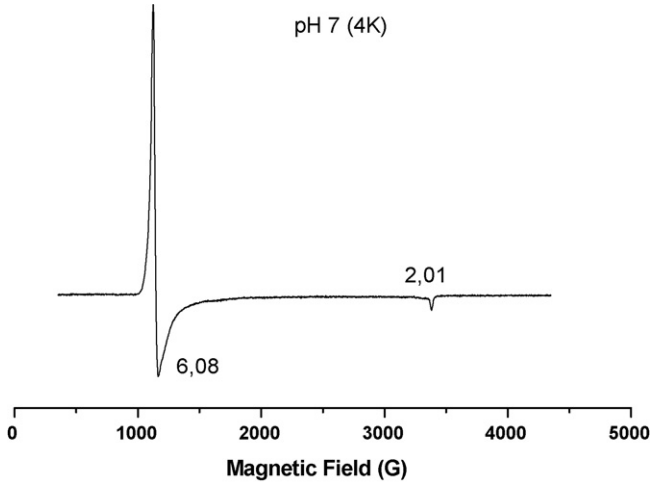

Fig. 2. Electron paramagnetic resonance spectrum of *Glossoscolex paulistus* hemoglobin with the *g*-values indicated. *Conditions*: 20 mM Tris–HCl buffer at pH 7.0 ($T = 4$ K). *EPR conditions*: modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 4.0 mW; microwave frequency, 9.4784 GHz.

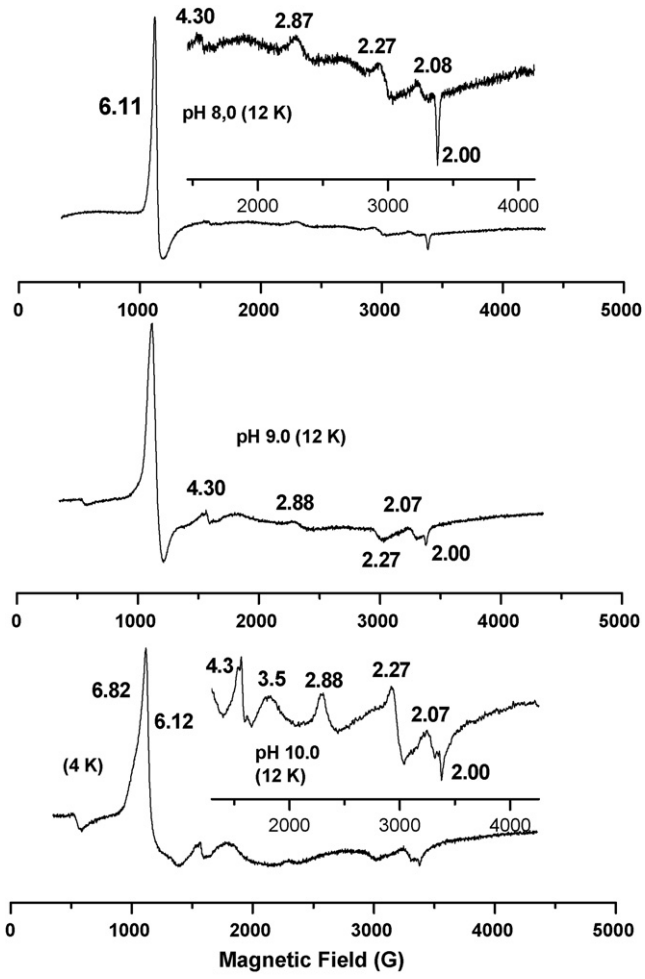

Fig. 3. Electron paramagnetic resonance spectra of *Glossoscolex paulistus* hemoglobin with the *g*-values indicated. *Conditions*: 20 mM Tris–HCl buffer; HbGp at pH 8.0 ($T = 4$ K showing insert with $T = 12$ K), HbGp at pH 9.0 ($T = 4$ K showing insert with $T = 12$ K), HbGp at pH 10.5 ($T = 4$ K showing insert with $T = 12$ K). *EPR conditions*: modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 4.0 mW; microwave frequency, 9.4780 GHz.

spin hemichrome species, also called hemichrome B or Type II hemichrome according to Walker classification, thus confirming the assignment of this species obtained from our UV–vis results. In fact, the *g*-values of the heme groups depend on the relative orientation of two imidazole rings and on the orientation of these imidazoles with respect to the porphyrin plane [54,55]. However, it is evident that the aquomet–hemichrome transition is not total, since the axial high-spin signal attributed to aquomet species remains in the EPR spectra, even after greater alkalization (Fig. 4), as already had been discussed previously in the present article in the UV–vis measurements section.

On the other hand, with higher alkalization an intense and broad peak appears in the spectrum around *g*-value 3.5. This peak has been attributed to the g_z-value of a low-spin heme species ($S = 1/2$). Unfortunately, it is not possible to identify from our spectra the g_x and g_y peaks due to the significant width of these spectral lines. This species would still be a bis-histidine species, such as the first hemichrome (the "pure" low-spin hemichrome), but their histidine ligands present a dif-

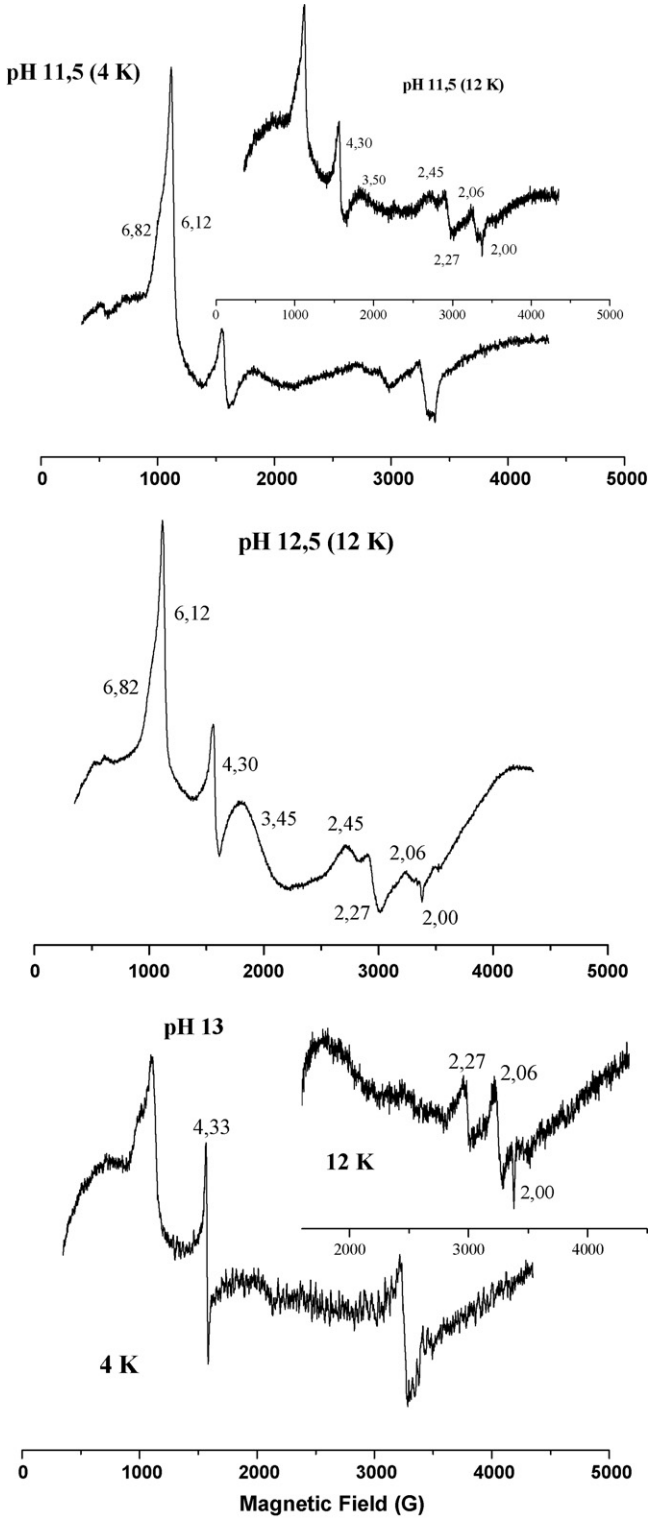

Fig. 4. Electron paramagnetic resonance spectra of *Glossoscolex paulistus* hemoglobin with the *g*-values indicated. *Conditions*: 20 mM Tris–HCl buffer. *EPR conditions*: $T = 12$ K, modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 4.0 mW; microwave frequency, 9.4794 GHz.

ferent spatial orientation. Thus, the orientation of the imidazole planes from the histidine ligands would be modified to originate a reciprocally perpendicular orientation. Therefore, in this new hemichrome, the planes of the imidazoles from the histidine axial ligands are in mutually perpendicular planes, while these orientations are reciprocally parallel in the "pure" low-spin hemichrome. This heme configuration with peculiar orthogonal spatial orientation of the axial ligand has been called highly anisotropic low-spin (HALS) or strong g_{max} species. According to Walker classification [12,15], this hemichrome is also denominated Type I hemichrome.

Subsequently, in the more alkaline pH values studied in the present work (Fig. 4), it was observed a significant anisotropy increase of the axial high spin peak, indicating that the high-spin species present in the more alkaline medium loosed symmetry, at least partially. This evidence is in agreement with the UV–vis results, indicating a representative presence in equilibrium of a pentacoordinate ferric species. Furthermore, a very intense signal around $g = 4.3$ is observed in the higher pH values, which has been identified in other ferric proteins and it has been assigned to non-heme iron centers [56,57].

The HALS hemichrome is probably a pre-requisite to the formation of the pentacoordinate ferric species, since the reciprocally orthogonal orientation to the axial ligand planes represents a weakness of the respective coordinated bounds. Indeed, McGarvey [58] attributes this configuration change to the Jahn-Teller effect, implying that this process decreases the orbital integral superposition between the metal and the axial bounds, increasing the ligation length. This weakness would decrease the stability of these ligands in the first coordination sphere of iron originating the rupture of at least one histidine from the iron ion, promoting the pentacoordinate ferric species formation. Rieger [59] has reported that Type I hemichrome presents an apparent near-degeneracy of the d_{xz} and d_{yz} orbitals that is consistent with various heme protein crystal structures, which showed that the two axial ligands lie in perpendicular planes because of the similar orbitals overlap of the d_{xz} and d_{yz} with the orbitals from the ligands.

The g-value at 2.45 (Fig. 4) could be associated to the unusual configuration $(d_{xz},d_{yz})^4(d_{xy})^1$, which corresponds to the Type III hemichrome [12]. This species would be originated from a drastic polypeptide influence on the porphyrin ring of the previous hemichrome species, triggering a pronounced ring distortion

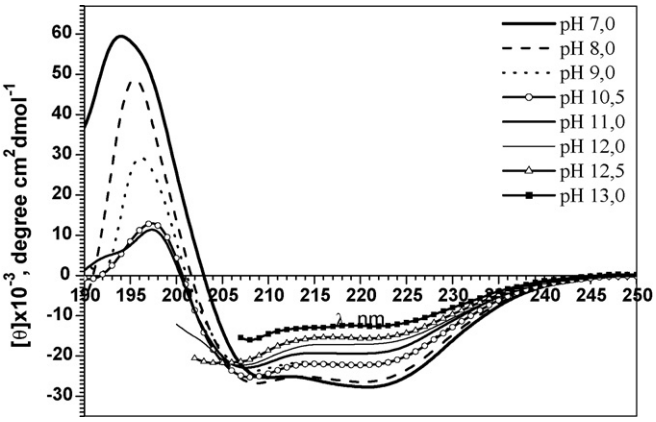

Fig. 6. CD spectra of *Glossoscolex paulistus* hemoglobin as function of pH. *Conditions*: protein concentration was 0.22 mg mL^{-1} (ε_{415} = 3.13 mL mg^{-1} cm^{-1}).

that modifies intensely the orbital superposition, changing the highest occupied molecular orbital (HOMO). In this way, the appearance of this species suggests a significant protein conformation change associated to an initial denaturation process, since this configuration occurs only in very alkaline conditions (pH values 11.5 and 12.5) (Fig. 4). Fig. 5 represents a simplified sequence of ferric species formation, based on our EPR results, as function of the medium alkalization.

3.3. CD measurements

The Far-UV CD spectrum of HbGp at pH 7.0 (Fig. 6) is characteristic of proteins having α-helices as a primary contribution to secondary structure. Upon alkalization CD spectra show a gradual loss of such α-helix content (Fig. 6). This fact can be inferred from the analysis of the intensity loss of the band at 222 nm. This spectral region is very used in the literature to evaluate the polypeptide structure [55,60]. The pH transition between 7.0 and 10.5 promotes a secondary structure loss of 19%. The pH value of 10.5 corresponds approximately to the pH that originates the hemichrome-pentacoordinate species transition, in agreement with UV–vis and EPR results, suggesting the importance of two factors to this process: (i) the mechanical changes in the polypeptide arrangement, which may provoke tension in the E and F helices, directly correlated to the heme;

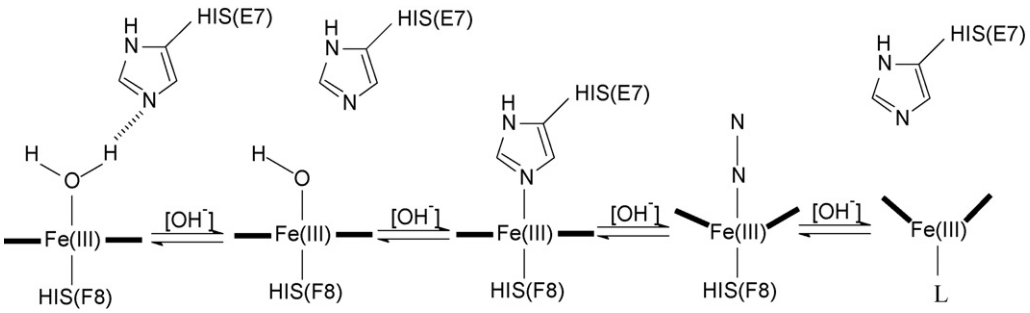

Fig. 5. The broad lines represent the side view of heme surfaces and the fourth model indicates the change of orientation of the distal histidine in relation to the heme, i.e., the side view of the distal histidine coordinated to the ferric center constituting the hemichrome HALS.

(ii) the water solvent accessibility in the heme pocket, which affects the accessibility of various ligands in the first coordination sphere of the metal, as well as destabilizes the important hydrogen bonds formed between the axial ligands and the globin structure.

4. Discussion

The results of the present work demonstrate the complexity of the equilibrium of HbGp ferric species as function of pH in alkaline medium. The transition aquomet–hemichrome-pentacoordinate species is not trivial, because the appearance of new species as function of the medium alkalization does not necessarily means the total elimination of the precursor species, i.e., the transition between hemoglobin species is incomplete. Furthermore, it was observed that the hemichrome species is, in fact, a complex equilibrium between different spatial arrangements of bis-histidine species, differing in the porphyrin ring conformations as well as in imidazole ring orientations.

Previous reports, which used only optical spectroscopies, suggested the existence of a high-spin hemichrome species, but this is not the case [22,23]. Our data show that *HALS* hemichrome, which is the bis-histidine complex that has the highest g, presents also $S = 1/2$. Indeed, the g-value 3.5 cannot be assigned to a high-spin ferric heme species. The *HALS* hemichrome is a pre-requisite to the pentacoordinate species formation, but the coexistence of these species would difficult their characterization via optical spectroscopies. Our analysis emphasizes the relevance of EPR techniques in for the improvement of studies of heme species equilibrium. The present results are in agreement with other works in literature regarding heme proteins species equilibrium, since the complexity of the heme species equilibrium is a typical characteristic of heme proteins, especially hemoglobins [61]. Unfortunately, despite of the high relevance of the use of EPR in studies focused on ferric species of hemoglobins, small number of works regarding giant extracellular hemoglobins (erythrocruorins) has employed this technique.

It is important to notice that the pK value determination for the transition between heme species is not an elementary procedure, because different species appear almost at the same pH value, i.e., the increase of the concentration of new species not necessarily means the complete disappearance of the previous species. It is a result of the complex influence of the protein oligomeric structure on the transitions that occur in the heme pocket. In fact, the polypeptide influence is very significant in hemeproteins, especially in the case of giant extracellular hemoglobins, due to their extraordinary molecular masses, which are higher than 3.1 MDa [19].

The sequence of HbGp species formation in alkaline medium is similar to the species formation sequence in acid medium [8]. However, the transition mechanism involved in alkaline medium is quite different. The transitions are more drastic in alkaline medium in conditions near to the neutrality, such as pH 8.0, and less intense in pH values that are more distant of the neutrality. The significant presence of a pentacoordinate species with histidine as fifth ligand, which does not occur in acid medium, is indicative of this higher resistance of one of the iron-histidine ligations in alkaline conditions. On the other hand, the representative presence of the line at $g = 4.3$, that is less intense in acid medium, suggests significant difference between acid and alkaline mediums with respect to the unfolding mechanism associated to the ferric heme species transitions.

In acid medium, the initial process of species transition is more gradual with the removal of the neutrality conditions [8]. Probably, this difference is related to the acid isoelectric point (pI) of the giant extracellular hemoglobins. The pI of this protein is approximately 5.5, implying that, at pH 7.0, an excess of negative charges occurs. In this way, a weak alkalization could be enough to originate a great electrostatic repulsion between the negative charges on the protein surface. This electrostatic repulsion, in turn, could promote the rupture of intra- and inter-subunits contacts, favoring the coordination of the distal histidine to the metal, originating the hemichrome species. This process would be accentuated for the oligomeric dissociation, that is very intense at pH 9.0, explaining why the fact of the heme species transition is more pronounced in alkaline medium when compared with the acid medium, in less distant conditions from the neutrality. Therefore, the acid medium would not originate enough anionic sites to promote a significant acid oligomeric dissociation as function of the weaker electrostatic repulsion between amino acid residues of the polypeptide chains as compared with the alkaline medium (5.5).

The dissociation process of the oligomeric structure is an important pre-requisite to ligand exchange in the first coordination sphere of the ferric center. It occurs because dissociation increases the water solvent accessibility in the heme pocket, which, in its native state at pH 7.0, is highly hydrophobic. The stability of the HbGp aquomet ferric species, for example, that is the predominant species at pH 7.0, is maintained by strong hydrogen bonds between the water ligand coordinated to the ferric metallic center and the distal histidine residue. The water solvent accessibility increase, decreasing the hydrophobicity of the heme pocket, weakens the hydrogen bond weakness, thus favoring the loss of the water ligand as function of the new hydrophilic interactions that are originated in the heme pocket. In a similar way, the medium alkalization could also promote the proton loss of the water ligand. A hydroxyl anion could then become the ferric center sixth ligand, which, usually, is an unstable ligand due to absence of a hydrogen bond that stabilizes this sixth ligand. This process would be simultaneously accompanied by the distal histidine coordination to the iron, originating the hemichrome species, due to rupture of intra- and inter-chain contacts as function of pH changes. Therefore, the displacement of the water ligand from the distal histidine is favored by the loss of intra- and inter-subunits contacts upon pH modification, allowing the distal histidine movement towards the metallic center.

In this way, all transition between heme species are associated with the increase of water solvent accessibility in the heme pocket as function of the oligomeric dissociation originated from pH changes. This fact illustrates the relevance of the heme hydrophobicity in controling and selecting ligands that can access and coordinate to the metal center. This property is also very important for the native oxy-HbGp species in maintaining

the original ferrous oxidation state, which is able to coordinate the molecular oxygen.

Previous reports evaluated the influence of ionic surfactants on the alkaline oligomeric dissociation and the consequent autoxidation of the ferrous oxy-HbGp species at pH values 7.0 and 9.0 [62,63]. The equilibria of ferric species shown in those articles are similar to the equilibrium obtained in the present work. Similar equilibria was also found for the native and reconstituted isolated d monomers [64,65], which suggests that the steric influence of the polypeptide chains is as important as the increase in water solvent accessibility, since the monomers present a heme pocket more accessible to the solvent than the whole protein and present the same tendency. The aquomet–hemichrome-pentacoordiante species formation sequence seems to be a common feature for various hemeproteins, when the original species presents the ferric oxidation state. On the other hand, oxy-hemichrome-pentacoordinate species formation sequence occurs when the first species shows the native ferrous oxidation state.

Moreover, in the interaction surfactant–HbGp, it was demonstrated that the electrostatic contribution is the predominant influence in the surfactant–hemoglobin interaction when compared with the hydrophobic contribution [62,63]. This effect would be due to the acid isoelectric point (p*I*) influence of the HbGp in this interaction, corroborating our results that show the decisive role of the acid p*I* of this hemoglobin. In this context, the *HALS* hemichrome could be considered a very important step in the heme species formation sequence because its formation seems to be a pre-requisite to the loss of the sixth ligand coordinated to the metallic center, i.e., the loss of the coordination of, at least, one histidine ligand. This point could be associated to various aspects of the structure–function relationship of heme proteins, in general, and, especially of the giant extracellular hemoglobins, requiring more studies to improve the understading of such relation. The influence of the ionic surfactant on the native ferrous species (oxy-HbGp) at pH 7.0 led to the formation of species similar to those obtained in drastic pH values, as in alkaline, which are discussed in the present work, as in acid medium, evaluated in previous report [8]. It is possible that the oligomeric structure of HbGp reacts in similar ways to different medium perturbations. In any case, pH changes and surfactant influences always induced oligomeric dissociation, unfolding and heme ferric species transition, even when the initial species is the highly conserved native ferrous oxy-HbGp species. This behavior is quite different from that observed from tetrameric hemoglobins, which, depending on the surfactant concentration, can promote folding and, consequently, inhibition of the hemoglobin autoxidation process [66,67].

Furthermore, it is important to notice that the EPR technique was a very convenient tool, able to detect the various hemichrome species, which coexist in equilibrium in alkaline medium. Therefore, it is clear that the HbGp hemichrome species characterized via UV–vis measurements represents a complex equilibrium involving species with very similar, but not equal, spin states, and, thus, cannot be distinguished by optical spectroscopies. Indeed, the majority of the hemoproteins UV–vis spectra are assigned to π–π^* bands, such as Soret and Q bands, implying that the evaluation of the first coordination sphere of iron from these bands is an indirect method. In opposite, EPR probes directly the coordination center, being more sensitive to its modifications. This complex equilibrium of hemichrome species could be a common property to several heme proteins, especially hemoglobins, and only would be conveniently evaluated for each case with a more widespread use of EPR spectroscopy. We believe that the research regarding hemichrome ferric species could be improved significantly with similar approaches.

5. Conclusions

The present work emphasizes the complexity of the equilibrium involving ferric heme species of HbGp as function of pH. Initiating the spectroscopic measurements from neutral conditions, the medium alkalization promotes a complex transition between different species due, especially, to the drastic influence of the dissociation of the extraordinary molecular mass of this giant extracellular hemoglobin, involving 180 polypeptide chains, and the polypeptide unfolding of each polypeptidic subunit. It was observed that the hemichrome species observed through UV–vis spectroscopy is a complex equilibrium involving different axial ligand orientations and spin states. Probably, the *HALS* hemichrome is a precursor species of the pentacoordinate ferric species due to the labilization of axial histidines coordination, which should favor the rupture of, at least, one of these ligations. Furthermore, the acid p*I* of giant extracellular hemoglobins seems to be an important factor to promote the heme species transitions, being associated to the different processes of species transition in alkaline medium as compared with the heme transitions in acid conditions, in agreement with previous work [8]. Probably, the higher number of anionic sites on the protein surface provokes a more pronounced unfolding of the oligomeric arrangement as function of a more intense electrostatic repulsion in pH values small removed from the neutrality. This explains the more significant alkaline oligomeric dissociation as compared with the oligomeric dissociation observed in acid medium. However, in more alkaline pH values, the mechanism is distinct and the reduced presence of the water ligand in the fifth coordination site of the metal, when compared with very acid conditions [8], suggests a different unfolding mechanims. Possibly, this is associated to an important influence of the acid p*I* on the HbGp oligomeric assembly, which would be associated to the important aspects of the giant extracellular hemoglobins structure–activity relationship. New studies are being developed in order to improve the understanding of this relation as well as the difference between acid and alkaline denaturation processes.

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