Ligand changes in ferric species of the giant extracellular hemoglobin of Glossoscolex paulistus as function of pH: correlations between redox, spectroscopic and oligomeric properties and general implications with different hemoproteins

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ABSTRACT: The present review is focused on the relationship between oligomeric and heme properties of HbGp, emphasizing the characteristics that can be generalized to other hemoproteins. This study represents the state-of-the-art with respect to the approaches for investigating giant extracellular hemoglobins as well as the correlation between oligomeric assembly alterations and their consequent changes in the first coordination sphere. A wide introduction focused on the properties of this hemoglobin is developed. Indeed, this hemoprotein is considered an interesting prototype of blood substitute and biosensor due to its peculiar properties, such as resistance to autoxidation and oligomeric stability. Previous studies by our group employing UV-vis, EPR and CD spectroscopies have been revised in a complete approach, in agreement with recent and relevant data from the literature. In fact, a consistent and inter-related spectroscopic study is described propitiating a wide assignment of "fingerprint" peaks found in the techniques evaluated in this paper. This review furnishes physicochemical information regarding the identification of ferric heme species of hemoproteins and metallic complexes through their spectroscopic bands. This effort at the attribution of UV-vis, EPR and CD peaks is not restricted to HbGp, and includes a comparative analysis of several hemoproteins involving relevant implications regarding several types of iron-porphyrin systems.

KEYWORDS: extracellular hemoglobin, ferric heme, ligand changes, optical absorption spectroscopies, EPR, pH.
INTRODUCTION

Globins are heme-containing proteins 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 [1].

The superfamily of hemoglobin is widely encountered in all life forms. Within this superfamily, mammalian hemoglobins, which act as oxygen transporters, constitute a $\alpha_2\beta_2$ heterotetramer. It is interesting to note that mammalian hemoglobins have been the most studied in terms of their function, structure and allosteric effect. On the other hand, primitive vertebrates and invertebrates have various types of hemoglobin that appear in monomer, oligomer or polymer form. Those hemoglobins present remarkably different quaternary structures and several properties of oxygen affinities. Indeed, a number of crystal structures of invertebrate monomeric, oligomeric and polymeric hemoglobins are available, and all these hemoglobins demonstrate quite different assemblage when compared to mammalian hemoglobins [2].

Hemoglobins have been extensively studied in order to clarify important questions concerning their function. Among the different hemoglobins, the annelid ones are considered of great interest regarding the structure-activity relationship, since these hemoglobins are giant extracellular aggregates, which present interesting structural and functional peculiarities. In fact, the understanding of the mechanisms associated with the different structural units that constitute a complex supramolecular arrangement and that communicate to each other, promoting high efficiency and regulatory control of the biological processes, is still a great scientific challenge [3].

In many annelids, oxygen transport relies on giant extracellular respiratory proteins (~3.6 $\times$ 10$^6$ Da), known as either erythrocrurins or hexagonal bilayer hemoglobins (HBL Hbs). Erythrocrurins are readily retained in the vascular system as freely dissolved entities. Each complex has a large oxygen-binding capacity and subunits can be arranged to allow cooperative oxygen binding and additional regulatory features that enhance oxygen transport [4].

Symmetrical arrangements of multiple subunits are observed in invertebrate giant extracellular respiratory proteins. These include the copper-containing hemocyanins from arthropods and mollusks, and heme-containing respiratory proteins such as those found in annelid worms. The most prevalent of these annelid complexes are the erythrocrurins hexagonal bilayer hemoglobins (HBL) [5]. Indeed, among the four types of existing respiratory proteins (hemocyanins, hemerytrins, chlorocruorins and hemoglobins) the hemoglobins are the most widely distributed in vertebrate and invertebrate animals [6].

Although invertebrates 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 [7]. Invertebrates possess hemoglobins with various architectures regarding the quaternary structure, with their appearance ranging from monomer to multimer composing the hexagonal bilayer ("bracelet model"). Vertebrates have hemoglobins with the usual quaternary structure, $\alpha_2\beta_2$ tetramer [7, 8]. Investigation of the relation between structure and function in invertebrate hemoglobins is expected to provide an important key to revealing the comprehensive oxygen-binding molecular mechanisms of hemoglobin [8]. However, compared with vertebrate hemoglobins, much less is known about the relations between their physiological functions and their molecular structures at the atomic level [7]. Furthermore, the potential applications of giant extracellular hemoglobins as blood substitute and biosensors reinforce the relevance of a more detailed analysis regarding these hemoproteins.

The giant extracellular hemoglobins represent the summit of complexity of the heme proteins that carries oxygen [9]. This is due to their extraordinary supramolecular masses (about 3.6 Mda), high cooperativity, redox and oligomeric stabilities, etc. These relevant properties have aroused the interest of various research groups, resulting in significant effort to employ this class of hemoglobin as blood substitute, i.e. artificial oxygen carrier [10–12]. In fact, Strand and co-workers [10] have proposed that erythrocrurins are useful model systems for developing therapeutic blood substitutes due to their extracellular nature, large size and resistance to oxidation.

Hirsch and co-workers [11] have suggested that the hemoglobin of Lumbricus terrestris (HbLt) in the ferrous form, at neutral pH, exhibits oxygen affinity and cooperativity similar to human hemoglobin. This fact has motivated the development of initial trials with HbLt as a model system of vertebrate hemoproteins, such as human hemoglobin. HbLt is a suitable model system due to the extraordinary redox and oligomeric stabilities of this hemoglobin, which does not require the respective research to be conducted under rigorous laboratory conditions. Other advantages of this hemoglobin class are its natural character and structural stability, together with its low probability to promote immunogenic responses, since cell membranes are not present and, unlike tetrameric hemoglobins, it does not undergo sub-unit dissociation upon dilution [13, 14].

Moreover, the high autoxidation resistance is considered a fundamental pre-requisite to this kind of physiological application [11, 15]. Harrington and co-workers [12] have studied these natural acellular polymeric hemoglobins that provide oxygen transport and delivery within many terrestrial and marine invertebrate organisms with the premise that these hemoproteins may serve as models of therapeutic hemoglobin-based oxygen carriers.
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The giant extracellular hemoglobin of Lumbricus terrestris (HBLT)

This fascinating protein system is associated with a very complex structure-activity relationship involving a great number of polypeptide chains. In this context, it is important to notice that the hemoglobin of the annelid Lumbricus terrestris (HBLT) has been considered the most studied erythrocytine. Indeed, HBLT was the first protein reported to be crystallized in 1840 by Hunefeld, was one of the first proteins investigated in Svedberg’s initial ultracentrifugation experiments, and an early molecular subject of electron microscopy [5]. The quaternary structure of this protein presents approximately 180 polypeptide chains, involving 144 globin sub-units 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 (Linkers chains) have only structural function. The oligomeric assembly is organized as two superposed hexagonal disks in what is 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 sub-units are more directly related to the hexagonal vertices of the “bracelet”. Each one of the “bracelets” has twelve dodecamers, i.e. each hexagonal site is associated with one dodecamer, each constituted by three tetramers abcd. In turn, each abcd tetramer is constituted by one abc trimer, the subunits of which are connected by disulfide bounds, and one d monomer. On the other hand, the Linker subunits that occur mainly in a central position of the “bracelet” present four kinds of chains (L1, L2, L3, L4), the amino acid residue sequences of which have been recently determined [18].

Therefore, the dodecamer is a fraction (1/12) of the whole protein, being associated then with (abc)dL, where L stands for the Linker chains. 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 [19], which denotes the high complexity of the allostery character of this protein associated with its quaternary structure.

In any case, understanding the effects of this complex structure on the cooperative behavior of giant extracellular hemoglobins is still a great challenge. In fact, cooperativity originates from different mechanisms in Lumbricus terrestris hemoglobin compared to vertebrate hemoglobins [19]. Strand and co-workers [10] 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 bound ligand corresponds to a rather crowded distal pocket, allowing for the inference of a representative impact on 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 with aromatic residues at B10, where the chains b and d present a residue of tryptophan, the a subunit has a residue of phenylalanine and the c chain presents a residue of leucine. This fact must be associated to the resistance to autoxidation found in the giant extracellular hemoglobins, denoting the implications between the very complex oligomeric assembly around the heme pocket and the redox properties of the ferrous ion, which are intrinsically correlated with the ligands present in the first coordination sphere of this metallic center.

The giant extracellular hemoglobin of Glossoscolex paulistus (HBGP)

The hemoglobin of Glossoscolex paulistus (HbGp) belongs to the same class as HBLT and these hemoglobins exhibit very similar structural characteristics [20–22]. In fact, Cabral and co-workers demonstrated that the monomer d of the hemoglobin of Glossoscolex paulistus presents 55% of sequence identity when compared with the correspondent d chain of HBLT. Glossoscolex paulistus is an annelid found in Araras, Rio Claro and Piracicaba, which are cities in Sào Paulo state in Brazil [23]. HBGP is a giant biopolymer that shows a minimum molecular mass of 3.1 MDa [24] and a complex assembly with a hexagonal double-layered (HBL) oligomeric structure [25, 26] that dissociates in alkaline and acid media [27–29]. This hemoprotein shows interesting features regarding natural selection, such as its adaptation to sulfide-rich environments [26, 30]. In this context, it is worth
noting that several giant extracellular hemoglobins, such as that from pogonophoran Oligobrachia mashikoi, have been studied for the structural influence of the sulfide-binding sites, which are considered determinant to the stability of the quaternary assembly of the giant hemoglobins [31]. Indeed, alternative mechanisms have been elaborated, proposing that the metallic cations, such as ions $Zn^{2+}$, are intrinsically related to the formation and stability of the sulfide bindings. These sulfide bindings would be mediated by zinc ions discovered in the crystal structure of tubeworm hemoglobins, implying a possible structural correlation between these ions and cysteine residues of the linker chains [31–33].

In the past few years, the research on the structural properties of HbGp has improved significantly the comprehension of several aspects of its structure-function relationship through spectroscopic studies. It is important to note that the protein medium conditions 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 [34–36]. Recent results have suggested that protons do have a very important function in the assembly of the HbGp in its integral state, which explains, at least partially, this decisive structural influence of pH changes [36, 37].

Studies focused on the kinetics of autoxidation of the ferrous form of this hemoglobin (Oxi-HbGp) have demonstrated that the pH changes constitute a determinant factor in initializing an intense oxidation process. This process is characterized by the loss of the radical anion superoxide ('O$_2^-$) from first coordination sphere of iron and the consequent appearance of ferric species [38, 39]. In this way, the own sixth ligand of iron, which is molecular oxygen, promoting oxidation of the metallic center, originates various kind of ferric species, depending on the pH of medium. Actually, the oxygenated forms of myoglobins and hemoglobins are easily oxidized to their ferric (met) forms [40].

Hemoglobins with low oxygen affinity have been reported to exhibit a high rate of autoxidation, showing that the oxidation rate seems to be inversely proportional to oxygen affinity [41]. In this way, the increase in autoxidation rates observed in Oxy-HbGp [38, 39] is probably associated with a decrease in oxygen affinity and, concurrently, an increase in the water solvent accessibility into heme pocket, especially around the distal site [42]. All hemoglobins present a heme pocket relatively isolated from the aqueous solvent, generating a significant hydrophobic character to the regions immediately proximal to the heme. Nevertheless, this general property seems to be highly pronounced in giant extracellular hemoglobins since direct contact with the blood solvent is a factor that favors heme oxidation. It is possible that the evolutive mechanisms of adaptation has contributed to the formation of a hemoglobin with extraordinarily high total mass in order to propitiate a more pronounced apolar shielding to the ferrous center, avoiding a more effective oxidation.

Although autoxidation is inevitable in nature for all oxygen-binding heme proteins, the met-Hb content of freshly drawn blood is usually maintained within 1–2% by virtue of a strong reducing environment [43]. It is important to note that autoxidation is a phenomenon of clinical and chemical interest, affecting the life of the erythrocyte and also being very useful in the elaboration of prototypes of blood substitutes [44].

Several discussions have been focused on the controversial point of the mechanism of reaction involved in the autoxidation process of hemoproteins. Shikama and co-workers [43, 45–50] have analyzed in detail this complex topic and, apparently, the spectroscopic and kinetic evidences have suggested that the internal sphere oxidation is the prevalent mechanism when compared with the external sphere one in most of the hemoprotein systems. Furthermore, several studies have demonstrated that the distal pocket is decisive to stabilizing the oxygen molecular bind to the iron center in the sixth site of coordination [51–54]. In this way, perturbations in the protein medium, such as pH changes, would be sufficient to alter the sensitive arrangement of the conformations of important residues of the globin subunits, especially the residues of amino acids localized around heme pocket.

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 [55, 56]. This difference between the iron oxidation states is an example of the oligomeric implications of the properties of the first sphere of coordination in giant hemoglobins. Therefore, a direct correlation between the characteristics of the first coordination sphere and the protein assembly is evident. Probably, the higher hydrophobic character of the well-conserved ferrous form precludes a higher permeability of water solvent into heme pocket, making the oligomeric dissociation process difficult. In fact, the highly hydrophobic character of the heme pocket is maintained by the presence of apolar amino acid residues around the iron-porphyrin, which is affected by the aqueous solvent permeability increase.

Alkaline oligomeric dissociation is associated with a simultaneous autoxidation process as a function of the water solvent accessibility increase into heme pocket, which, in 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 [57]. In this way, a kind of dissociation-autoxidation cooperative effect occurs when medium perturbations originate an initial process of oligomeric dissociation.
It is important to note that in acid medium, it is also possible for some oligomeric dissociation to occur that is similar to that peculiar to alkaline medium [58]. 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 [9, 58]. Mainwaring and co-workers [58] determined that the pI of HbLt is 5.5. Indeed, Arndt and co-workers [59] 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 the repulsion of negative charges that are in excess within the protein, changing its structure but preserving its absolute molecular mass. Thus, when this decrease of cohesion of the polypeptide chains 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 in determining various properties of the structure-activity relationship, since it is associated with the intensity of compaction of the oligomeric assembly and, consequently, is related to the level of hydrophobic isolation of the heme pocket.

The ligand binding kinetics of non-vertebrate hemoglobins are strongly influenced by the structure of the heme cavity, particularly the size and polarity of residues occupying the distal portion that exert sterio and dielectric effects [7]. 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 mechanisms have been found to contribute to the modulation of oxygen affinity in allosteric hemoglobins [60].

Further of the oxidation process, the water permeability increase into heme pocket originates a very interesting and complex transition between different species through change in the ligands coordinated to metal 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 in ferric species this ligand is the water molecule.

**THE FERRIC FORM OF THE GIANT EXTRACELLULAR HEMOGLOBIN OF GLOSSOSCOLEX PAULISTUS**

Ferric forms of hemoglobin are physiologically inert to further oxygenation, but several subsequent side reactions in the Hb autoxidation reaction may interfere or merge into other biochemical pathways, including the formation of a hemichrome whose physiological role is still a very controversial topic [40]. The bis-histidine complex can be involved in ligand binding in the in vivo reduction of met-Hb, in Heinz body formation in vertebrate organisms, and in NO scavenging [61]. In fact, it has been suggested that hemichrome can be involved in a complex process of protection from oxidation attack [62].

The highly hydrophobic character of the heme pocket in the hemeproteins, especially the erytrocruorins, is a decisive factor in stabilizing the water molecule as the sixth ligand of the metallic center when oxidation takes place in neutral medium (pH ~ 7.0). This occurs because the hydrophobic environment is not attractive to interact with a polar molecule, such as H2O, and, consequently, when this ligand entry in the heme pocket, it would be immediately attracted by the cationic coordination center, which is the ferric ion. So, 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 and/or sites 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 heme. Indeed, the dielectric constant increase, as a function of the higher permeability of the aqueous solvent into 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. In contrast, the breakage of inter- and intra-subunit contacts becomes the distal histidine free to “bore deep” into heme and to compete with the water for the coordination of the ferric ion. Considering that the histidine is a stronger ligand than water molecule for coordinating the ferric ion due to its higher σ-donor character (more intense Lewis basicity), this facility of movement by E-helix is decisive to the distal histidine displacing the water ligand from the metallic center. Actually, Vergara and co-workers explain that the formation of the β-hemichrome is associated with a scissors-like movement of the EF helices in the β-units, which makes the distance between distal and proximal histidines close enough to form the endogenous bis-histidine complex [40]. The endogenous hexacoordination would be also favored by sliding of the β-heme plane, which moves towards the exterior of the heme pocket [40]. The flexibility and possibility of spatial conformation changes constitute decisive influences to determine the physicochemical properties of the
iron-porphyrin systems and hemoproteins [63–70]. The understanding of the properties of this protein system is very important to bioinorganic chemistry, since the bis-histidine coordinated heme centers are involved in a great number of hemoproteins, such as cytochrome-containing systems, which is the case of cytochromes b of mitochondrial Complexes II and III and of chloroplast cytochrome b6f [71].

Thus, any medium perturbation that provides an oligomeric change is an agent that can provoke some ligand exchange at the first coordination sphere of the ferric ion due to the structural inter-relationship between polypeptide chains and the iron-porphyrin. Indeed, the processes 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 pressure, and (v) changing the pH of medium [72]. Usually, these medium perturbations affect the protein assembly and promote protein unfolding through the breakage of inter- and intra-subunit contacts (which are weak interactions, such as van der Waals contacts). So, each polypeptide chain becomes less crowded and freer, in its molecular dynamic, to originate movements independently of the other subunits. Consequently, the distal histidine is free to coordinate the metallic center of the complex. Furthermore, in a species of synergic mechanism, this unfolding process usually promotes a polarity increase into heme pocket that destabilizes the water coordination, favoring more intensely the ligand exchange.

Studies focused on the complexity of the equilibrium between hemoprotein ferric species as a function of pH have been developed, especially with hemoglobins [73]. These studies have shown that 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 relationships. Indeed, the pH changes that originate an intense dissociative process for giant extracellular hemoglobins, especially in alkaline medium, lead to hemoglobin conformational changes, including allosteric phenomena, implying that the evaluation of the pH influence is a relevant step to improving the understanding of the normal protein folding and its function [36, 74].

SPECTROSCOPIC PROFILES OF THE FERRIC SPECIES OF HBGP AS FUNCTION OF pH

The association between different spectroscopic techniques has propitiated wide information about the physicochemical properties of each configuration of the hemoproteins. Although several works focus on this topic, interesting data presented on the literature are not correlated to each other. In this way, the correlation between some data obtained from different instrumental techniques must be considered an important tool for the characterization of the physicochemical profile of the hemoproteins and iron-porphyrin complexes. For this review, we selected the very appropriate connection between UV-vis, EPR and CD spectrosopies, as indispensable prerequisites to an adequate interpretation of the very complex hemoprotein systems.

UV-vis measurements

UV-vis spectrum at pH 7.0 is a typical evidence of the presence of a single aquomet species. This species is encountered at this pH in a great number of hemoglobins, being identified by a Soret band with maximum wavelength at 405 nm, which presents a slight asymmetry in its lower wavelengths, a Q band at 500 nm and a LMCT band at 630 nm [75–79] (Fig. 1).

The decrease in pH values from neutrality generates a gradual process of spectral change. The decrease from pH 6.0 to pH 3.0 causes an absorbance increase and a small red-shift of the Soret band simultaneously, indicating hemichrome formation, which corresponds to a bis-histidine species, which is characterized by Q bands at 532 and 574 nm [80–84] (Fig. 1). Thus, this spectral change is associated with the distal histidine coordination to the...
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ferric ion. The movement of the distal histidine imidazole towards the metal center is probably the result of a slight alteration in the globin arrangement in the heme pocket as a function of pH changes. It is important to note that not only pH changes can generate hemichrome formation, but that such formation also occurs with other types of medium perturbations, such as surfactant interaction. Indeed, a great number of recent articles demonstrate unequivocally the significant formation of bis-histidine species, even at pH 7.0, due to the drastic polypeptide perturbation that the interaction between the different kinds of surfactant cause in the protein sites [34, 85, 86].

Approximately at pH 3.0, a drastic spectral transition is observed, which is evidenced by a Soret band blue-shift, when compared with the spectrum at pH 7.0, associated with a simultaneous decrease of absorbance, which indicate the formation of a pentacoordinate species as previously described by several authors [87–115] (Fig. 1).

In addition to the mono-histidine pentacoordinate species, there is probably a significant presence of other ferric pentacoordinate species. In fact, the heme group can dissociate of the globin, losing direct contact with the polypeptide chains. In this situation, the heme group can, but not obligatorily, leave the heme pocket. In any case, it is important to register that the group heme, which is free of any strong bonds with the polypeptide chains, is still a pentacoordinate species. For this reason, it is very difficult to differentiate these two coexistent ferric pentacoordinate species, since their spectroscopical characteristics are very similar. The free heme group would present a water molecule as ligand in the fifth coordination site. In fact, ferric hemes are more stable, when present with 5 or 6 ligands, which is usual to metallic centers with electronic configuration $d^8$, such as ferric ion (Fe(III)).

In alkaline medium, a similar tendency of species formation is observed, denoting that HbGp reacts in a quite proximal way to the medium conditions when compared with the acid medium (Fig. 2). It is important to note that the eventual presence of the hydroxymet-hemoglobin species would be just an intermediary step of the transition between aquomet and hemichrome species and its presence must occur in a minimum concentration. Furthermore, the characteristic wavelength of hydroxymet-hemoglobin species is significantly superposed by the bands peculiar to the other species, which are more representative in the respective medium. It is clear that the

Fig. 2. UV-vis electronic absorption spectra of whole hemoglobin of Glossoscolex paulistus between 250 and 700 nm in alkaline medium with insert in the main spectral regions regarding the Soret band; and the Q and ligand-to-metal charge transfer (LMCT) bands, respectively

<table>
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<tr>
<td>7.0</td>
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<td>8.0</td>
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<td>9.0</td>
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<td>10.0</td>
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<td>12.0</td>
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<td>12.5</td>
<td>364 (shoulder), 396, 536 (shoulder), 577, 608</td>
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<td>13.0</td>
<td>364 (shoulder), 385, 580, 610</td>
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Fig. 3. Comparative schematic representation of the intensity maximum of the main spectral bands in (A) acid and (B) alkaline media, respectively
transition aquomet-hemichrome-pentacoordinate species is a kind of preferential sequence of ferric species formation to the acid medium as well as the alkaline one. It is possible to note this fact in Fig. 1, which demonstrates a similar profile of transitions when compared with Fig. 2 that is associated to the acid medium. Interestingly, the alkaline transition between the ferric species aquomet and hemichrome occurs in a pH value less distant of neutrality than this transition in acid medium. In this way, the stability of the ferric aquomet species in acid medium is significantly higher than in alkaline medium, which is probably associated with the acid pI of this class of hemoglobin. Therefore, the electrostatic contacts between the charged amino acid residues is much more affected with small pH changes in alkaline medium when compared with similar changes in acid conditions. This occurs due to the representative increase of electrostatic repulsion that affect the intra- and inter-subunit contacts, favoring the hemichrome formation in conditions that are more proximal of the neutrality. Furthermore, it is possible to note that the hemichrome-pentacoordinate species transition is much more resolved and drastic than this transition in alkaline medium, which could also be associated to the acid isoelectric point (pI) (Fig. 3).

It is important to note that this sequence of ferric heme species formation is relatively similar to a great number of hemoproteins. In this way, the basis of the UV-vis bands assignment is very proximal to the attribution presented in this review regarding HbGp. This is due to the electronic absorption bands in the ultraviolet-visible range being basically constituted by intra-ligand electronic transitions of the porphyrin ring (B and Q bands) and ligand-to-metal charge transfer (LMCT). In this way, in many cases, the similar oligomeric properties as well as the ligand properties of the residues that are near to the heme induce the occurrence of the heme transitions in a significantly proximal way. In this way, the present discussion focused on the band assignment of UV-vis spectra of hemoproteins presents a representative general character to the other hemoproteins. This fact is noted by Table 1, which lists several articles that have focused on different hemoproteins presenting similar spectroscopic bands, which indicate the presence of the same ferric heme species.

### EPR measurements

Frozen-solution EPR spectra constitute a powerful technique that is highly suitable for developing studies focused on the first coordination sphere of iron-porphyrin model metallic complexes as well as the hemoproteins. Indeed, EPR is an excellent tool for characterizing the electronic ground state of ferriheme complexes at approximately 4.2 K, and has been demonstrated in several works [116]. In any case, in order to avoid saturation of the signal, sometimes additional measurements at approximately 12 K are necessary, which decreases slightly the time of life in the excited state, favoring the relaxation process and minimizing the probability of saturation phenomenon. Indeed, the association between measurements developed at 4 and 12 K is especially interesting for observing the spectral peaks assigned to the different types of ferric low-spin species which, in the case of met-hemoglobins, corresponds to the hemichrome species that present higher intensity to the analysis made at 12 K when compared with those developed at pH 4.2.

Figure 4 presents the EPR spectrum of whole aquomet-hemoglobin at pH 7.0, demonstrating that this medium condition generates a significantly “pure” species. Figures 5, 6 and 7 demonstrate the ferric heme transitions obtained in acid medium, where the peculiar peaks referent to the different types of hemichrome can be identified. In fact, the EPR spectrum at pH 7.0 is characteristic of an axially symmetric high-spin species with g_|| and g_perp around 2.0 and 6.1, respectively (Fig. 4). In this case, only one component is observed, indicating the existence of one high-spin configuration assigned to the aquomet species.

<table>
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<th>λ, nm</th>
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<td></td>
<td>405, 500, 540, 633</td>
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<td>404.9, 500.2, 580, 630.5</td>
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<td>411, 541, 576, 600</td>
<td>Feis, 1994 [155]</td>
</tr>
<tr>
<td>Hemichrome</td>
<td>530, 565</td>
<td>Bogumil, 1995 [156]</td>
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<td></td>
<td>535, 565</td>
<td>Antonini, 1981 [153]</td>
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<tr>
<td></td>
<td>530, 560</td>
<td>Tsuruga, 1998 [81]</td>
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<tr>
<td>Pentacoordinate</td>
<td>403, 645</td>
<td>Ilari, 2002 [100]</td>
</tr>
<tr>
<td></td>
<td>602</td>
<td>Spagnuolo, 1994 [157]</td>
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<td></td>
<td>400, 600 e 605</td>
<td>Boffi, 1999 [158]</td>
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<td></td>
<td>393–402</td>
<td>Tada, 1998 [89]</td>
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<td></td>
<td>–400</td>
<td>Ikeda-Saito, 1992</td>
</tr>
<tr>
<td></td>
<td>390–395</td>
<td>Quilin, 1993 [76]</td>
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<tr>
<td></td>
<td>397</td>
<td>Kamimura, 2003 [77]</td>
</tr>
<tr>
<td></td>
<td>395</td>
<td>Gilles-Gonzalez, 1994 [78]</td>
</tr>
<tr>
<td>pH 7.0–395, 506, 642</td>
<td>Bogumil, 1995 [156]</td>
<td></td>
</tr>
<tr>
<td>pH 10.5– 405, 502, 640 with shoulder at 370</td>
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LIGAND CHANGES IN FERRIC SPECIES OF HEMOGLOBIN OF GLOSSOSCOLEX PAULISTUS

The acidification causes the appearance of several species, whose spectra are shown in Figs. 5 and 6 as a function of the pH value. Overall, three low-spin species are formed in the spectra (see \( g \approx 2 \) region of the spectrum, insert of Fig. 3), which are assigned to hemichromes, i.e. bis-histidine complexes. In hemoproteins, it is well established that bis-histidine hemes having two axial histidine ligands display similar EPR spectra with \( g \) values between 2.9 and 3.6. The \( g \)-values of these complexes depend on the relative orientation of the two imidazole rings and on the orientation of these imidazoles with respect to the porphyrin plane [117, 118]. These hemichromes show different electronic configurations: two of them are in the more usual spin state \((d_x^2d_y^0d_z^0)\), and are characterized by \( g \)-values at 2.87 and 2.27 (Fig. 3) and by \( g \)-values around 3.47 (Fig. 4); the third hemichrome presents an unusual electronic configuration \((d_x^0d_y^0d_z^1)\) with a resonance at 2.45 (Figs 3 and 4). The \( g = 4.3 \) line in Fig. 4 has been observed in other ferric proteins and it has been assigned to non-heme ferric residues [119, 120]. Consequently, it will not be further considered in our discussion.

Between the hemichromes with more usual electronic configurations, the first species (\( g \)-values of approximately 2.87 and 2.27) presents mutually parallel imidazole ring orientation (Type II or B-hemichrome) [68], whereas in the second species (\( g \)-values around 3.47) those rings are in an orthogonal conformation (Type I hemichrome or HALS (highly anisotropic low-spin)) [68, 121–127]. It is worth mentioning that only through EPR spectroscopy was it possible to distinguish these hemichromes.

It is also possible to note the presence of an asymmetric axial spectrum, which is characteristic of pentacoordinate ferric heme species (Figs. 6D and 7A,B), corroborating the interpretation of the data obtained with UV-vis spectroscopy. Figures 8 and 9 present similar ferric heme transitions encountered in the alkalization of the medium. In agreement with UV-vis data, it is possible to infer that a lightly alkaline medium is more effective in promoting the heme transitions when compared with a slightly acid medium, which must be associated to the acid pI of this hemoglobin. Furthermore, it is clear that a more defined hemichrome-pentacoordinate transition occurs in acid medium as compared with alkaline medium.

The identification of different species of hemichrome is only possible by employing EPR spectroscopy as a function of the proximity of the energetic levels. In this context, it is very relevant to emphasize the work developed by Walker and co-workers, who have made an extraordinary contribution in this area through their study and classification of model complexes and hemoproteins regarding the different types of reciprocal orientation among axial ligands and the spatial conformations of the porphyrin ring [68–70, 128, 129]. These possible arrangements are decisive to determining the spin state and redox properties of the metallic center in hemoproteins. In the classification proposed by Walker and co-workers [6, 69, 128, 129], Type I complex shows a usual spin state \((d_x^2d_y^0d_z^0)\) and a reciprocally orthogonal orientation of the heterocyclic planes of the axial ligands. Type II complex presents also spin state \((d_x^0d_y^0d_z^1)\), but parallel orientation between the respective ligand planes. Type
**Fig. 6.** Electronic Paramagnetic Resonance (EPR) spectra of whole met-hemoglobin of HbGp obtained in acid medium at 4 and 12 K (pH values 6.0; 5.0; 4.0; 3.0)

**Fig. 7.** Electronic Paramagnetic Resonance (EPR) spectra of whole met-hemoglobin of HbGp obtained in acid medium at 12 K (pH values 2.5 and 2.0)
III complex has an unusual spin state \( (d_{xy})^4(d_{xz},d_{yz})^1 \), 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 from the imidazole group. Walker and co-workers have also discussed the significant occurrence of bis-histidine species in cytochromes and have compared their geometric and electronic structures [69].

Zaric and co-workers [130] have investigated the factors that determine conformations of the imidazole axially coordinated to heme in hemoproteins by analyzing 693 hemes in 432 different crystal structures from the Protein Data Bank (PDB). In this collection, 65 conformations were bis-histidine-ligated hemes, illustrating the considerable occurrence of this species in heme proteins. The authors [130] argue that 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 effect that favor the perpendicular form. In heme model systems, the orientation of axial ligands can depend on crystal field stabilization effects or on steric effects caused by substituents on axial ligands and on the porphyrins. 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 [131] performed quantum chemical (DFT) calculations on heme model systems with non-substituted Fe-porphyrin core for the different orientations of the axially coordinated imidazoles. Their results indicated that perpendicular orientation (Type I hemichrome) can be explained by steric effects caused by propionic groups of porphyrin ring and by the histidine backbone.

In this context, it is important to register the contribution of McGarvey [132], who described a more detailed explanation for the HALS (Type I) hemichrome formation considering that imidazoles planes, which were initially in a parallel arrangement, adopt a perpendicular configuration, which is similar to a tetragonal distortion. In this way, the heme avoids the occurrence of a "classic" Jahn-Teller effect, with the axial ligands preferring the perpendicular

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**Fig. 8.** Electronic Paramagnetic Resonance (EPR) spectra of whole met-hemoglobin of HbGp obtained in alkaline medium at 4 and 12 K (pH values 8.0; 9.0; 10.0; 11.5)
orientation, which shows that smaller orbitals overlap with the iron orbitals than the parallel orientation, thus causing a smaller splitting of iron $d_{\sigma}$ orbitals. In this way, this “non-peculiar Jahn-Teller effect” does not increase significantly the distance between the axial ligands and the metallic center, but generates a similar decrease in the overlap between the orbitals involved in the respective coordinations, avoiding a more intense orbital interaction. Rieger [133] 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 the similar orbitals overlap to $d_{xz}$ and $d_{yz}$.

The heme transitions involving the different ferric species are evidently of incomplete character, i.e. the appearance of a new species does not mean the total consumption of the precursor species. This fact denotes an important characteristic of equilibria associated with hemoproteins, especially hemoglobins, which is the great complexity between the constituent species of their equilibria [134]. In fact, Svistunenko and co-workers have demonstrated that the coexistence of distinct species is an inherent characteristic of hemoglobin equilibria, being that the degree of complexity and coexistence depend on the medium conditions [134]. The present review illustrates this tendency, denoting that only the pH changes are sufficient to generate intense alterations in the first coordination sphere, but that these transitions are not completed. In this way, we can conclude that the coexistence of ferric species of hemoglobin is not a simple and conventional equilibrium and that this equilibrium occurs as a consequence of the drastic mechanical influence of the polypeptide chains on the heme pocket.

The different kinds of hemichromes present an important transition to a ferric pentacoordinate species when the pH of the medium is already relatively distant of neutrality. Actually, the rhombic high-spin signals suggest the formation of ferric pentacoordinated species [40, 76, 136–140], in agreement with the analysis obtained from UV-vis spectroscopy.

**Fig. 9.** Electronic Paramagnetic Resonance (EPR) spectra of whole met-hemoglobin of HbGp obtained in alkaline medium at 4 and 12 K (pH values 12.5 and 13.0)
It is important to notice the great variability of species of hemoproteins that present the same tendency of formation of ferric pentacoordinate species. This fact can be observed, for example, in the interesting articles of Vergara and co-workers [40] and Giordano and co-workers [135] that are focused on hemoglobins of Antarctic and Arctic fish species, respectively. Furthermore, besides HbGp [139–140], mutants of myoglobin [76, 138], peroxidases [137], a flavo hemoglobin [100], the Scapharca inequivalvis hemoglobin [136], and others hemoproteins present the same propensity to form ferric pentacoordinate species, which denotes that this tendency is a characteristic inherent to several hemoproteins associated to its structure-function relationship.

In this way, the global analysis of UV-vis and EPR spectra allows for the inference of the simplified sequence of heme species formed with the acidification or alkalization of the respective medium (Fig. 10). Interestingly, the species formed with both changes of the medium conditions generate similar configurations. This fact is caused by the decisive mechanical influence of the oligomeric arrangement as well as the properties of ligand of proximal and distal histidines.

**CD measurements**

Far-UV circular dichroism spectra for HbGp as a function of pH are presented in Fig. 11. At pH 7.0, the CD spectrum is characterized by positive bands at 195 nm and two negative bands at 208 and 222 nm, which are assigned to significant α-helix content expected in most hemoglobins. Upon acidification at pH values below 5.0, a considerable decrease of both bands centered at 195 and 222 nm is detected, which is related to a
representative decrease of helical content. It is important to note that the stability of the aquomet species at pH 7.0 is directly dependent on the high level of hydrophobicity into heme pocket. In this way, the medium acidification must promote an increase of the water solvent accessibility into heme pocket, destabilizing the hydrogen bond between the water ligand and the distal histidine (E7). This hydrogen bond is a decisive influence that contributes intensely to the stability of the coordination of the water molecule as sixth ligand of the ferric center. Thus, the loss of the α-helical content makes the whole hemoglobin more permeable to the external solvent, favoring the accessibility of the solvent into heme pocket, which, \emph{a priori}, presents elevated hydrophobic character. Therefore, the alteration in the degree of compaction of the polypeptide chains allows the region around the iron-porphyrin to increase its dielectric constant, promoting the breakage of the hydrogen bond between water ligand and distal histidine that induces the loss of this sixth ligand.

The absolute values of residual ellipticity at 195 and 222 nm tend to decrease with the increase in the distance from neutrality in acid and alkaline mediums. However, there is only one point that disturbed this behavior and it is around pH 5.0 (Fig. 11). In this way, maximum value of residual ellipticity at 195 and 222 nm occurs approximately at pH 6.0. If it is known that the pH 7.0 represents the medium condition in which HbGp presents its whole state regarding its quaternary structure, this pH should represent an optimum condition to the structure-function relationship of HbGp, \emph{i.e.} this medium would be quite proximal of the “native” conformation of HbGp that is functional in the biological conditions. Therefore, this higher α-helix content in the range of pH 6.0–5.0 could only be assigned to the proximity with the acid ipI of HbGp. In fact, this pH range, which represents the acid ipI of this class of giant extracellular hemoglobins, would be associated with a more compact state of “aggregation” since the contact between the polypeptide subunits would be maximum due to the occurrence of its zwitterionic point. In this way, there is not any prevalence of one kind of charge (negative or positive) around ipI, precluding a more intense electrostatic repulsion involving the inter- and intra-chain contacts (Fig. 11).

The relationship between the increase of water solvent accessibility in the heme pocket and the loss of secondary structure would indicate that the pentacoordinate ferric species could be an initial step of the denaturation process. Madura and co-workers [141] argue that, in most vertebrate hemoglobins, the changes in protein tertiary structure are induced by either ligand binding or changes in oligomeric assembly at the heme neighborhood. Nevertheless, it is interesting to note that the changes observed for \emph{L. terrestris} and \emph{G. paulistus} hemoglobin

![Fig. 11. Dichroism Circular (CD) spectra of whole met-hemoglobin of HbGp obtained in (A) acid and (B) alkaline media in the spectral range between 190 and 250 nm]
are much less drastic in the same conditions than those that occur with vertebrate hemoglobins. In other words, we can affirm that the giant extracellular hemoglobins require much more drastic pH values to generate the species that are produced in lighter conditions to the vertebrate hemoglobins.

It is possible to infer that the quaternary assembly of extracellular hemoglobins is less susceptible to the solvent permeability when compared with vertebrate hemoglobins. In fact, Hundahl and co-workers [142] assert that the small effects of water in the extracellular invertebrate hemoglobins may be correlated with the small surface-to-volume ratios in these high-molecular-weight proteins that pose a limit to water-accessible sites, and suggest smaller quaternary structural changes when compared to dimeric and tetrameric hemoglobins. Therefore, the extraordinary supramolecular mass of the erythrocruorins develops a kind of “protector effect” regarding the heme pockets, making the initial step of the denaturation phenomenon difficult. This analysis is in agreement with the proposal of several authors who argue that the influence of the hydrogen bond between the water molecule as sixth ligand and the distal histidine (E7) is decisive to the stability of the ligation water-ferric center as well as the consequent heme properties, such as electronic structure, ligand affinities, coordination number, redox potential and macrocycle conformations [143–147].

**IRREVERSIBILITY OF THE HEME TRANSITION AS FUNCTION OF PH CHANGES**

Studies focused on the possibility of reversibility of ferric heme transitions of HbGp have demonstrated that the modifications in the first coordination sphere and the neighborhood of the heme pocket are, at least partially, of irreversible character (Figs 12 and 13). In fact, analysis developed by EPR indicates the impossibility of a significant return to the original heme conditions [85]; this is corroborated by studies employing UV-vis which show that the typical spectral profile of met-HbGp at pH 7.0 is not regenerated after drastic transitions of pH, even returning the medium conditions to neutral value of pH. Furthermore, it is also evidenced by CD that the loss of secondary polypeptide structure is irreversible, characterizing a pronounced denaturation process. These evidences demonstrate that the mechanical influence of the polypeptide chains constitutes a decisive factor in determining the ligands that coordinate the ferric ion, implying that the equilibria associated to different pH values are not conventional equilibria due to the high degree of irreversibility of each ferric heme transition.

The literature demonstrates that the complexity of equilibria in ferric heme proteins as function of pH is a general reality in hemoglobins, myoglobins and cytochromes. In fact, any external agents which perturb the oligomeric arrangement of heme proteins, such as addition of surfactants, can induce irreversible transitions. This is a consequence of the decisive role of the polypeptide chains in the ligand changes in the first coordination sphere. In fact, the mechanical influence of the protein subunits constitutes the principal factor that determines the ligands coordinated to the ferric center. The original aquomet species at pH 7.0 is only stable when the polypeptide assembly is highly conserved, precluding, mechanically, the direct competition between the water and the distal histidine (imidazole) ligands. It occurs due to the fact that the imidazole of the lateral chain of distal histidine is a much better ligand to ferric ion when compared with the water molecule. However, in more drastic pH conditions, the mechanical influence of the polypeptide chains as a function of the spatial changes and the breakage of intra- and inter-subunit contacts is a determinant factor in originatning the labilization of one of the ferric ion-histidine ligations, generating a significant presence of pentacoordinate species. However, it is important to note that these transitions are not complete, which makes the precise determination of pK values a difficult work. It happens because the appearance of a new species does not mean the total

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**Fig. 12.** Electronic Paramagnetic Resonance (EPR) spectra of whole met-hemoglobin of HbGp obtained after drastic transitions from acid medium.
elimination of its precursor species. Furthermore, with the pH changes, several species are formed without the total absence of the original species (aquomet at pH 7.0). Therefore, the complexity of ferric heme species equilibria seems to be a general characteristic of the several heme proteins due to the kind of ligand available to coordinate the metallic center and their respective affinities to the ferric ion. In any case, only the employment of EPR is able to evidence the different types of hemichrome, being a fundamental technique in propitiating a more consistent study of the ferric heme species present in different medium conditions to heme proteins.

**INTERACTION OF OXY-HBGp WITH IONIC SURFACANTS**

Studies focused on the interaction between HbGp and ionic surfactants have demonstrated that the acid isoelectric point of HbGp (approximately 5.5) is an important factor in determining the intensity of interaction between HbGp and different ionic surfactants, such as cationic (CTAC) [34], anionic (SDS) [38, 39] and zwitterionic (HPS) [148] ones. In any case, the ferric species formed through autoxidation process induced by the surfactant-hemoglobin interaction are approximately the same as that obtained with only pH alterations. However, heme transitions that only occur at pH values relatively distant of neutrality, which is the case in hemichrome formation, can be formed by the decisive influence of the surfactant even at pH 7.0. It is important to register that studies focused on other hemoproteins have demonstrated the presence of similar species. In fact, the aquomet and hemichrome species have been found in several studies involving autoxidation and interaction with surfactants [149–152].

**CONCLUSION**

The oligomeric perturbations that occur with HbGp as a consequence of pH alterations or interaction with surfactants provide approximately the same ferric species independently of the initial species. In fact, initiating from ferric aquomet-species or ferrous oxy-species, the first transition is predominantly to the ferric hemichrome species. However, it is important to note that this hemichrome species corresponds to a complex equilibrium involving several ferric bis-histidine species, which is detected only by employing EPR spectroscopy, since the spectral differences obtained by optical spectrosopies do not present pronounced differences. Subsequently, the general tendency is the ferric heme transition between hemichrome species and pentacoordinated species. These two fundamental transitions are due basically to the decisive mechanical influence of the oligomeric chains on the first coordination sphere. An extraordinary volume of data from literature, involving mainly hemoglobins, myoglobins and cytochromes, indicate that the hemoproteins present a general tendency as well as spectroscopic similarities, independently of their structural differences. In any case, some characteristics of the heme transitions encountered in HbGp are very typical and constitute a fingerprint of its properties when compared with other hemoproteins, such as the absence of hydroxyl species, the representative stability of aquomet species in acid medium, the low stability of aquomet species in alkaline medium, and the elevated level of coexistence between a great number of different species in a wide pH range. Knowledge of these similar physicochemical properties is a determinant prerequisite to the understanding of each specific difference between the various hemoproteins. Probably, the properties as ligand of proximal and distal histidines and, mainly, the decisive mechanical influence of the polypeptide chains constitute the principal factor determinant of the heme transitions. This explains, at least partially, the widespread occurrence of hemichrome and pentacoordinate ferric species in several types of hemoproteins when these globins are submitted to some medium perturbation. Similarly, this fact is associated to the significant irreversibility of the heme
transitions and the complexity of the consequent ferric species equilibria. This process is intrinsically related to several mechanisms of hemoprotein denaturation. It is important to note that these transitions occur independently of whether the initial heme is a ferrous or ferric species, emphasizing the relevance and the general character of this chemical behavior to various hemoprotein systems, not only to HbGp. In this way, we believe that a more detailed spectroscopic analysis, mainly employing EPR, is fundamental to understanding the correlation between the changes that occur in the periphery of the hemoprotein with the alterations inherent to its principal biological site, which is the heme group.

REFERENCES

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LIGAND CHANGES IN FERRIC SPECIES OF HEMOGLOBIN OF GLOSSOSCOLEX PAULISTUS
