18

Giant Extracellular Hemoglobin of *Glossoscolex paulistus*: Excellent Prototype of Biosensor and Blood Substitute

Leonardo M. Moreira et al.*
Departamento de Engenharia de Biossistemas (DEPEB),
Universidade Federal de São João Del Rei (UFSJ)
Brazil

1. Introduction

Porphyrins and their metal complexes have been investigated for many years because the richness of the properties of these compounds is of interest to a wide range of scientific disciplines, from medicine to materials science (Figure 1). Metalloporphyrins in living systems play many functions that are essential for life, and the elucidation of both the geometric and electronic structures of these compounds is of extreme relevance to a detailed understanding of their roles in biological systems. Moreover, the possibility of mimicking the complex chemistry exhibited by metalloporphyrins in living organisms with synthetic models propitiates the possibility of exploiting then in a wide range of different applications, from medical diagnostics and treatments to catalysts and sensors (Walker, 2006).

The heme groups (iron porphyrins) sites are involved in a range of biological functions. These roles are developed through various biochemical processes, such as electron transfer (e.g., cytochromes *a*, *b*, *c*, and *f*), in which the heme cycle between low-spin Fe(II) and low-spin Fe(III) small-molecule binding and transport, catalysis, and O_2 activation (e.g. peroxidases and cytochromes P450), where high-valent iron centers are involved in several chemical reactions, such as hydrogen atom abstraction, hydroxylation, and epoxide formation (Figure 1). Heme sites are significantly different from non-heme iron sites in which the porphyrin ligand allows the delocalization of the iron d-electrons into the porphyrin π system. This distribution of electronic density changes the properties of the iron with respect to the flexibility of the central coordination site, the energetics of reactivity, and, consequently, to its biological function (Hocking et al., 2007).

Alessandra L. Poli[2], Juliana P. Lyon[3], Pedro C. G. de Moraes[1], José Paulo R. F. de Mendonça[1], Fábio V. Santos[4], Valmar C. Barbosa[5] and Hidetake Imasato[2]
[1]*Departamento de Engenharia de Biossistemas (DEPEB), Universidade Federal de São João Del Rei (UFSJ), Brazil*
[2]*Instituto de Química de São Carlos (IQSC), Universidade de São Paulo (USP), Brazil*
[3]*Departamento de Ciências Naturais (DCNAT), Universidade Federal de São João Del Rei (UFSJ),Brazil*
[4]*Campus Centro Oeste Dona Lindu, Universidade Federal de São João Del Rei (UFSJ), Brazil*
[5]*Instituto de Fìsica (IF), Universidade Federal do Rio de Janeiro (UFRJ), Brazil*

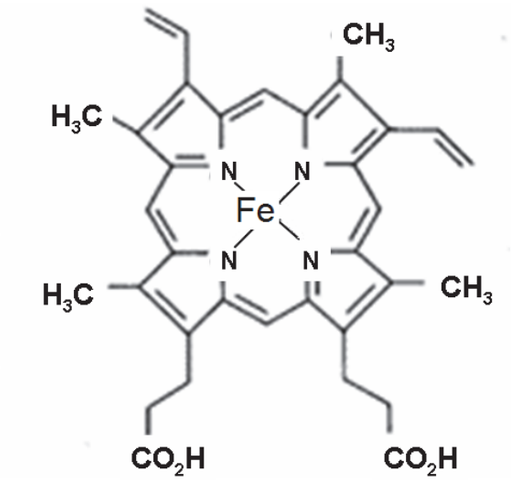
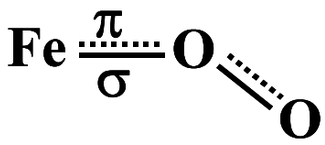


Fig. 1. Protoporphyrin IX (PpIX) demonstrating the ferrous íon as coordination Center and the nitrogens of the four pyrrolic rings acting as coordinating sites (Lewis basis).

The heme groups (iron porphyrins) sites are involved in a range of biological functions. These roles are developed through various biochemical processes, such as electron transfer (e.g., cytochromes *a*, *b*, *c*, and *f*), in which the heme cycle between low-spin Fe(II) and low-spin Fe(III) small-molecule binding and transport, catalysis, and O_2 activation (e.g. peroxidases and cytochromes P450), where high-valent iron centers are involved in several chemical reactions, such as hydrogen atom abstraction, hydroxylation, and epoxide formation (Figure 1). Heme sites are significantly different from non-heme iron sites in which the porphyrin ligand allows the delocalization of the iron d-electrons into the porphyrin π system. This distribution of electronic density changes the properties of the iron with respect to the flexibility of the central coordination site, the energetics of reactivity, and, consequently, to its biological function (Hocking et al., 2007).

The structure-activity relationship of iron-porphyrins as well as the activity-function relation of globins is still a great challenge to several researchers. Understanding the function of macromolecular complexes is related to a precise knowledge of their structure. These large complexes are often fragile high molecular mass noncovalent multimeric proteins (Bruneaux et al., 2008). This extraordinary hemoprotein system is widely distributed in nature, presenting slight differences between the several types of heme proteins. In spite of the various similar physico-chemical properties, the apparently small significant differences are responsible for a diversity of characteristics that becomes quite distinct the biochemical behavior of these proteins. In this way, the association of instrumental tools is essential to elucidate intricate aspects involving the structure-function relationship of these protein systems. By combining native mass and subunit composition data, structural models can be proposed for large edifices such as annelid extracellular hexagonal bilayer hemoglobins (HBL-Hb) and crustacean hemocyanins (Hc) (Bruneaux et al., 2008). Association/dissociation mechanisms, protein-protein interactions, structural diversity among species and environmental adaptations can also be addressed with these methods (Bruneaux et al., 2008). An example of these light structural differences that provoke significantly distinct functions is the case of the nitrophorins that are NO-carrying hemoproteins, being significantly different of the O_2-carrying hemoproteins, such as

hemoglobin (Figure 2). Nitrophorins constitute an example of this complex reality, since that these proteins are a group of NO-carrying hemoprotein encountered in the saliva of, at least, two species of blood-sucking insects, *Rhodnius prolixus* and *Cimex lectularius*, which present very elaborated physico-chemical properties deeply associated to its complex biochemical role (Berry & Walker, 2007; Knipp et al., 2007). These hemoproteins sequester nitric oxide (NO) that is produced by a nitric oxide synthase (NOS) present in the cells of the salivary glands, which is a protein similar to vertebrate constitutive NOS. NO is kept stable for long periods by ligation as sixth ligand of the ferriheme center. Upon injection into the tissues of the victim, NO dissociates, diffuses through the tissues to the nearby capillaries to cause vasodilatation, and thereby allows more blood to be transported to the respective site of the wound. At the same time, histamine, which causes swelling, itching, and initiates the immune response, is released by mast cells and platelets of the victim. In the case of the *Rhodnius* proteins, this histamine binds to the heme iron sites of the nitrophorins, hence preventing the victim`s detection of the insect for a period of time, which allows it to obtain a sufficient blood meal (Berry & Walker, 2007; Knipp et al., 2007). It is important to notice that great and crescent number of studies that employees porphyrin-like compounds in different chemical contexts denotes the extraordinary interdisciplinary and multidisciplinary characters of these macrociclic compounds. The applications of porphyrin-like compounds, metallated or not, in PDT (Moreira et al., 2008), catalysis, electrochemical studies, biomimetic studies, and others are a definitive fingerprint of the great biochemical and physico-chemical relevance of this chemical system.



(1.3)

Fig. 2. Iron-Oxygen bound, with the Oxygen molecule (oxygen-oxygen bound axix) presenting significant inclination in relation to the iron-oxygen bound axis.

2. Electronic properties of heme groups

The delocalization of the Fe d-electrons into the porphyrin ring and its effect on the redox chemistry and reactivity of these systems has been difficult to study by optical spectroscopies due to the dominant porphyrin π-π* transitions, which obscure the metal center (Hocking et al., 2007). In any case, the information obtained from Ligand-to-Metal Charge Transfer (LMCT) transitions can be accessed in several cases, mainly when this electronic band occurs above 600 nanometers. In this situation, it is possible to infer a higher number of relevant physico-chemical data from electronic spectra. Recently, Hocking and co-workers (Hocking et al., 2007) developed a methodology that allows the interpretation of the multiplet structure of Fe L-edges in terms of differential orbital covalency (i.e., differences in mixing of the d-orbitals with ligand orbitals) using a valence bond configuration interaction (VBCI) model. This method can be considered an interesting alternative to obtain significant information about the heme properties, principally when these data are not accessible through UV-VIS spectroscopy. In fact, when this methodology is applied to low-spin heme systems, this method allows experimental determination of the delocalization of the Fe d-electrons (Figure 3) into the porphyrin (P) ring in terms of both

PfFe *ó* and *ð*-donation and FefP *ð* back-bonding. We find that *ð*-donation to Fe(III) is much larger than *ð* back-bonding from Fe(II), indicating that a hole superexchange pathway dominates electron transfer (Hocking et al., 2007).

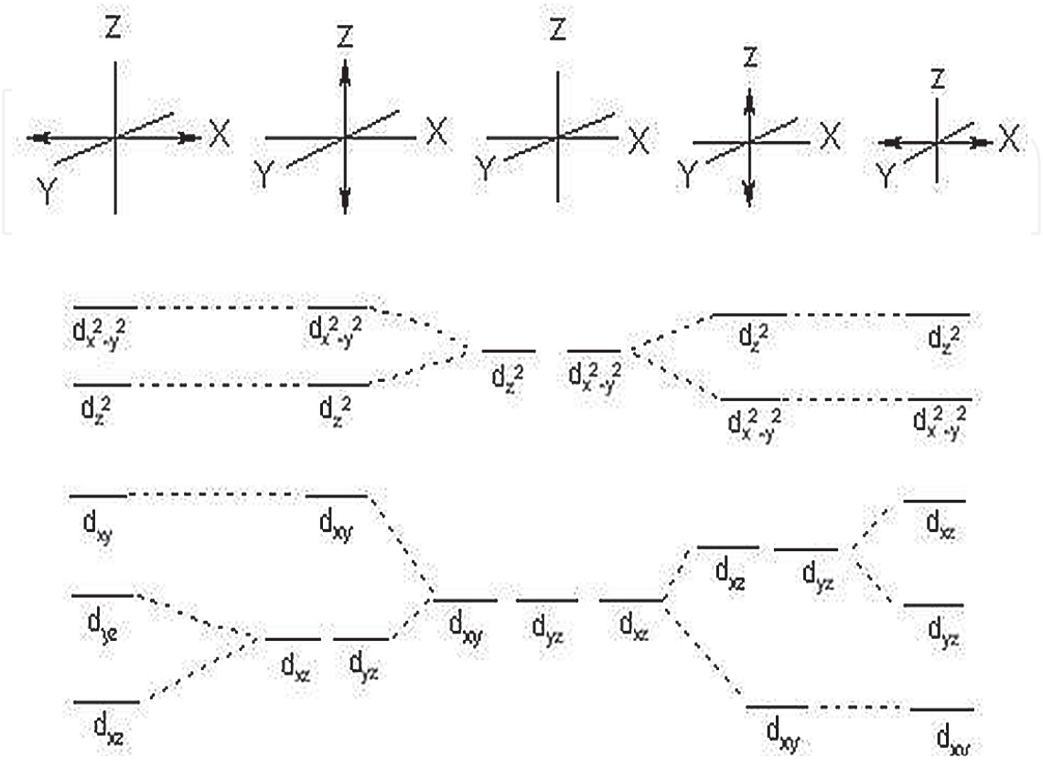


Fig. 3. 3*d* orbitals splitting related to octaedric complexes that present tetragonal and rhomboedric distortions complexos octaédricos devido às distorções. Right side: Assymetric distribution of d_{xz} e d_{yz} orbitals intensifies the Jahn-Teller distortions provoking the rhombic symmetry. The tetragonal symmetry is favored in the absence of steric precluding.

3. Hydrophobic isolation of the heme pocket in hemoproteins and the aqueous solvent role in the structure-activity relationship

Binding of water to hemoglobin is the determinant step in the mechanism of allosteric regulation (Pereira et al., 2005). An analytical method known as osmotic stress has been developed based on this inclusion/exclusion process for situations of low macromolecular concentrations. This methodology is being extensively applied to analyze the hydration water involved in the interaction of macromolecules (Pereira et al., 2005). Furthermore, the water action upon the hemoglobin structure is deeply associated to the native hydrophobic isolation inherent to the heme pockets of hemoproteins. This hydrophobic isolation limits significantly the access of aqueous solvent to the metallic center, which implicates in a more stable redox state as well as lower number of ligand changes of the first coordination sphere of the metallic center. Consequently, when the natural hydrophobicity of the native heme pocket is maintained, it is limited the occurrence of hemoglobin autoxidation (Figure 4), which would be accentuated by the presence of anionic ions in the heme pocket (Figure 5)

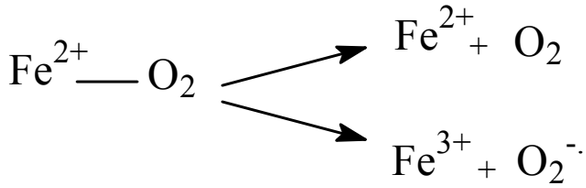


Fig. 4. Oxygen ligand exit from the first coordination sphere of the ferrous ion, which can occur as superoxide anion (autoxidation) or neutral oxygen molecule

4. pH influence on the oligomeric structure of hemoproteins

The effect of pH on biological systems has been widely investigated using various models to gain insights into the role of protons in modulating biochemical processes. Analysis of the stability of high protein aggregates using hydrostatic pressure (>250 MPa) to promote protein dissociation has shown that protein aggregation is strongly pH-dependent (Bispo et al., 2005). The ability of protons to cause protein conformational changes, including allosteric phenomena, means that the study of pH is important for understanding normal protein folding and function. In hemoglobins (Hbs), the role of protons in oxygen affinity (Bohr effect) has been extensively studied in the physiologic pH range and at extreme conditions. The cooperativity in ligand binding is also pH-dependent, with a decrease in cooperativity as pH increases. This behavior is responsible for the sigmoidal nature of the plot of Hb saturation versus oxygen pressure, with a tendency to assume a hyperbolic shape at alkaline pH (Bispo et al., 2005). These properties demonstrated the high sensitivity of the oligomeric structure of hemoglobins to the environmental changes.

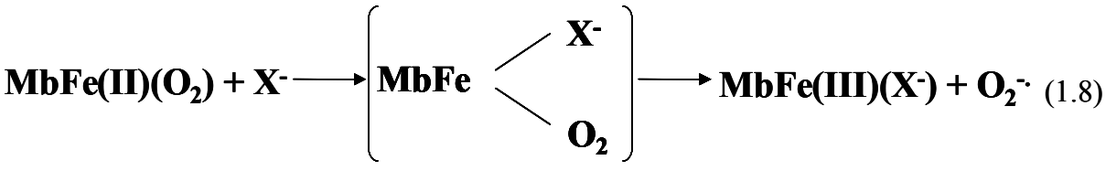


Fig. 5. Autoxidation mechanism favored by the presence of anionic ligand

5. The interface between redox and oligomeric properties of hemoproteins

Studies focused on the evaluation of redox potential of human hemoglobins have demonstrated a negative value of redox to the couple of *quasi*-reversible redox peaks, such as -0.38 V (*versus* SCE) in 0.1 M pH 7.0 PBS obtained through a Hb/gelatin/GCE system (Yao et al., 2006). Zhao and co-workers performed their direct electrochemistry with the formal potential of -0.032V for hemoglobin and system of ZrO_2 nanoparticles with heme proteins on functional glassy carbon electrode (Zhao et al., 2005). In fact, the determination of redox potential in hemoproteins is frequently associated to complex methodologies, since the polypeptide chains and the hydrophobic isolation of the heme pocket preclude the direct contact between the electrode and the main redox site of hemoproteins, which is the metallic center of heme (ferrous or ferric ion). These redox potentials strongly suggest that the heme group of several heme proteins, especially hemoglobins, would be easily oxidized in a short time interval, mainly if the accessibility of oxidant agents into heme pocket would not deeply precluded.Considering these redox potentials, it is plausible to infer that only the

hydrophobic isolation would not be able to avoid the oxidation of the ferrous ion. The limitation propitiated by the lateral chains of the aminoacid residues of the heme pocket neighborhood is not sufficient to maintain, at least, 95% of heme species in its ferrous form. In fact, in mammalian organisms, only 1% of ferric species is considered a normal physiological condition, being that the minimum of 99% is reached by the action of reductase enzymes, which limits significantly the concentration of ferric form in the organism. In any case, the redox potential of most hemoproteins would suggest a more representative percentage of oxidized heme species in the respective organisms. However, this fact does not occur as function, mainly, of the great compaction that constitutes the native state (wild configuration) of the hemoproteins, especially in hemoproteins with great supramolecular mass, which is the case of the giant extracellular hemoglobins. This high level of compaction of the globin chains limits pronouncedly the accessibility of ions into heme pocket. In fact, it is well established that the more intense accessibility of potential ligands to the metallic center is a decisive factor to improve the autoxidation rate (Figure 5).

Liu and co-workers (Liu et al., 1996) claims that the major difference between the Im-cyt and cyt *c* lies in their respective redox potential (-178 mV for Im-cyt *c* versus 260 mV for cyt *c*). In this context, the functional relevance of the axial Met80 ligand can be emphasized. In summary, the variation in the redox potentials of cytochrome *c* can be accounted for by differences in two effects: (a) the nature of the axial ligation to the iron; (b) the effects of the surrounding protein environment. The substitution of axial methionine by imidazole has been indicated to decrease the redox potential of cytochrome *c* by 160 mV. Since the imidazole ligated cyt *c* has a potential of 438 mV lower than the native cyt *c*, it appears that environmental factors may be most important. In fact, axial ligands provided by the side chains of His-18 and Met-80 as well as the covalently attached heme not only are essential for the structure and function of cytochrome *c* (cyt *c*), but they also play an important role in the folding process. It has been demonstrated by optical and NMR spectroscopy that one of the axial ligands in native oxidized cyt *c*, Met-80, dissociates more readily, and can be displaced by the intrinsic or extrinsic ligands, for instance, in the zinc cyt *c* used in the electron transfer studies or in the alkaline form of the protein (Shao et al., 1996). The high reduction potential of cyt *c* is also caused by exclusion of water from the heme environment by the surrounding hydrophobic and bulky amino acids. In Im-cyt *c*, apparent changes have happened to the heme hydrophobic pocket including heme-contact residues within 60 s helix, the region around Met80 and the lower left side of the molecule which is near be accounted for by changes in the secondary structures for example, the absence of 3_{10} helix from Tyr67 to Asn70, the type II turn form from Ile75 to Thr78 and distorted 50s helix from 51 to 54 (Liu et al., 1996).

Previous data focused on giant extracellular hemoglobins demonstrated that the initial protein unfolding provoked by interaction with low concentration of ionic surfactants promotes a surprising increase in the size of the supramolecular protein arrangement, probably caused by the discompaction of the chains. Interestingly, only after this slight polypeptide chains separation, it is possible to differentiate the physico-chemical and spectroscopic behaviors of each chain. Therefore, besides the protection against oxidation, the compaction would be the main cause of a very peculiar phenomenon, which is the equalization of heme groups, due to the intense compactness of the quaternary structure. This proposal is supported by several studies found on autoxidation kinetics that demonstrated that an initial loss of intra- and inter-chains contacts is a fundamental pre-requisite to the initial oxidation of the heme groups. The oxidation phenomenon has

demonstrated to be very dependent of the occurrence of some medium perturbation. For example, we can mention the pH changes, which, affecting the relation of ionic charges that involves the intra- and inter-chains contacts, decrease the compaction level of the quaternary arrangement of the protein. In fact, all factors that can perturb the global spatial configuration of the polypeptide chains can be considered as potential inductors of oxidation, since that the oligomeric alteration of the native form tends to originate a less compacted configuration. In this way, pH changes, surfactant addition and oxidant agents presence between others, constitute decisive influences that gradually decrease the native characteristics of the hemoglobin quaternary and tertiary configurations. Thus, an initial discompaction must to occur with concomitant increase of the protein size previously to a more representative unfolding process. This gradual process of loss of compaction would be the predominant phenomenon if the protein perturbation is small, which can occur when, for example, the surfactant concentration or the pH change is low (small distance of the neutrality). In more drastic processes, including drastic pH transitions and addition of high surfactant concentration, the discompaction is already accompanied by a very pronounced protein unfolding and until, in some cases, of an initial chains separation. Probably, in these drastic processes, the interaction of surfactants with the protein is a micelle-like phenomenon, being characterized by a significant concentration of the ionic surfactants molecules on each ionic site of opposite charge situated on the protein surface. On the other hand, low concentration of surfactants propitiates a more specific and individual interaction between the surfactant molecules and the sites of opposite ionic charge that are encountered on the protein surface.

6. The redox-dependent structure change in hemoproteins: Comparative analysis between ferrous and ferric forms

Many studies focused on understanding the structure-function problem in several hemoproteins, such as cytochrome *c*, have revealed that *ferricytochrome c* is different from *ferrocytochrome c* in several physical and chemical properties, including global stability, compressibility, molecular extent evaluated by low-angle X-ray scattering, hydrogen-exchange behavior and the chemical reactivity of specific groups (Feng et al., 1990). Therefore, the redox state change generates a sequence of relevant events that can alter drastically the protein activity. The cytochrome *c* protein favors the reduced form of its bound heme, which means that the heme binds more strongly to the protein in the reduced form and therefore makes reduced cytochrome *c* the more stable form. The higher structural free energy level of the oxidized proteins reveals itself when structural stability is measured, e.g., in equilibrium denaturation experiments which measure overall stability and, at higher resolution, in the hydrogen-exchange rates of individual hydrogens, which depend upon local unfolding reactions (Feng et al., 1990).

7. The iron ligands and the structural implications regarding the configuration of the first coordination sphere

The ligand affinities for O_2 and CO in monomeric hemoglobin (Hb) and myoglobin (Mb) are exquisitely modulated over wide ranges by relatively few residues within a largely conserved globular fold consisting of 7-8 helices with the heme wedged between helices E and F and ligated by His(F8) (Kolczak et al., 1997) (Figure 6). Various direct influences have been proposed to modulate the stability of the "ligated protein" in comparison with

unligated protein, including hydrogen-bond stabilization by the ubiquitous His(E7) of the bound O_2 (observed in neutron diffraction of MbO_2) (Figure 6), steric destabilization by Val (E11) for bound CO (observed by tilt or bend of Fe-CO in X-ray diffraction of MbCO or HbCO), and pocket polarity as determined by residues such as B10. A more indirect mechanism proposed to modulated ligand affinity in general is the control of the spacing between the F-helix and the heme, which must be significantly reduced in ligated form when compared with the unligated state (Kolczak et al., 1997).

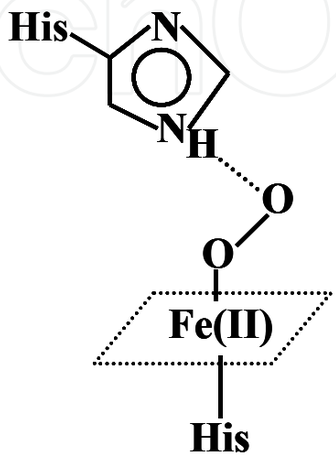


Fig. 6. Distal histidine E7 generating stability to the iron-oxygen bound through the formation of a hydrogen bound between the NH group of imidazole and the molecular oxygen.

8. Hemoglobins

Among the four types of existing respiratory proteins: (a) hemocyanins; (b) hemerytrins; (c) chlorocruorins; and (d) hemoglobins, the latter is widely distributed in the vertebrate and invertebrate animals (Arndt & Santoro, 1998). In the mollusks, the main oxygen carrier is the copper containing hemocyanin and extracellular hemoglobins (erythrocruorins) are restricted to two families of clams, Astartidae and Carditidae and one family of freshwater snails, the Planorbidae (Arndt & Santoro, 1998). The snail extracellular hemoglobins are multi-subunit proteins with reported molecular weights varying between 1.65 and 2.25_103 kDa. They contain 10 to 12 polypeptide chains of 175–200 kDa linked in pairs by disulfide bridges forming five to six subunits of 350– 400 kDa. Each of these chains comprises 10 to 12 heme binding domains based in its minimum molecular weight of 17.0–22.5 kDa (Arndt & Santoro 1998). This pattern is also observed in the branchiopod crustacean *Artemia sp* which contains 9 domains per polypeptide chain and corresponds to the multi-domain, multi-subunit structure reviewed by Vinogradov (Arndt & Santoro, 1998). This kind of structure denotes the relevance of the polypeptide contacts, which are decisive to determine the intensity of compaction of the hemoprotein, generating the tertiary and quaternary structure that are peculiar to each protein. Hemoglobin (Hb) occurs in all the kingdoms of living organisms. Its distribution is episodic among the nonvertebrate groups in contrast to vertebrates. Nonvertebrate Hbs range from single-chain globins found in bacteria, algae, protozoa, and plants to large, multisubunit, multidomain Hbs found in nematodes, molluscs and crustaceans, and the giant annelid and vestimentiferan Hbs comprised of globin and

nonglobin subunits. Chimeric hemoglobins have been found recently in bacteria and fungi. Hb occurs intracellularly in specific tissues and in circulating red blood cells (RBCs) and freely dissolved in various body fluids (Weber & Vinogradov, 2001).

9. Mammalian hemoglobins

Mammalian adult hemoglobin (HbA) is a tetramer of two Hb and two Hb subunits (Figure 7), which is produced in extremely high concentrations (340 mg mL^{-1}) in red blood cells (Gell et al., 2009). Numerous mechanisms exist to balance and coordinate HbA synthesis in normal erythropoiesis, and problems with the production of either HbA subunit give rise to thalassemia, a common cause of anemia worldwide (Gell et al., 2009). In this context, it is interesting to notice that Hematrocrit (Ht) levels higher or lower than the normal range can influence the physiological function and increase the risk of cardiovascular disease. The Ht level is indicative of the proportion of blood occupied by red blood cells, and is normally 40.7–50.3% for males and 36.1–44.31% for females (Sakudo et al., 2009).

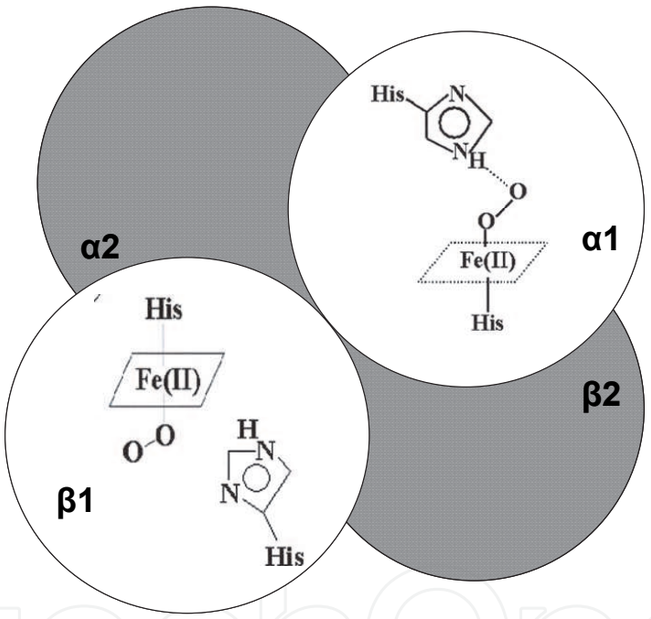


Fig. 7. Scheme of the contact $\alpha_1\beta_1$, which favors the inclination of the distal histidine of β chain of the human hemoglobin.

10. Extracellular hemoglobins

The hemoglobin from Biomphalaria glabrata is an extracellular respiratory protein of high molecular mass composed by subunits of 360 kDa, each one containing two 180 kDa chains linked by disulfide bridges, being that data regarding the structural and biochemical properties indicate that the multisubunit structure of this hemoglobin is compatible with a tetrameric arrangement (Arndt et al., 2003). However, there is a great number of extracellular hemoglobins that presents a much more complex quaternary structure, preseting a higher number of oligomeric subunits, which are called giant extracellular hemoglobins (HBLs).

11. Giant extracellular hemoglobins (HBLs)

Assembly of protein subunits into large complexes is an important mechanism employed to attain greater efficiency and regulatory control of biological processes. Annelid erythrocruorins pose key problems in the design of such large macromolecular assemblages. The extracellular nature and giant size of these molecules have made them ideal systems for a number of seminal investigations into protein structure (Royer et al., 2000). Natural acellular polymeric hemoglobins (Hb) provide oxygen transport and delivery within many terrestrial and marine invertebrate organisms. These natural acellular Hbs may serve as models of therapeutic hemoglobin-based oxygen carriers (HBOC) (Harrington et al., 2007). For instance, acellular Hb from the terrestrial invertebrate Lumbricus terrestris (Lt) possesses a unique hierarchical structure and a peculiar ability to function extracellularly without oxidative damage. Lumbricus Hb as well as Arenicola Hb is resistant to autoxidation, chemical oxidation by potassium ferricyanide, and have low capability to transfer electrons to Fe(III)complexes at 37°C. An understanding of how these invertebrate acellular oxygen carriers maintain their structural integrity and redox stability in vivo is vital for the design of a safe and effective red cell substitute. In fact, this hemoglobin presents positive redox potential (Harrington et al., 2007). Homotropic and heterotropic allosteric interactions are important mechanisms that regulate protein function. These mechanisms depend on the ability of oligomeric protein complexes to adopt different conformations and to transmit conformation-linked signals from one subunit of the complex to the neighboring ones (Hellmann et al., 2008). An important step in understanding the regulation of protein function is to identify and characterize the conformations available to the protein complex. This task becomes increasingly challenging with increasing numbers of interacting binding sites. However, a large number of interacting binding sites allows for high homotropic interactions (cooperativity) and thus represents the most interesting case (Hellmann et al., 2008). Giant extracellular hemoglobins are examples of very large and cooperative protein complexes. This class of hemoglobins is found in annelid worms that contain 144 oxygen-binding sites, such as the giant extracellular hemoglobins of *Lumbricus terrestris* and *Glossoscolex paulistus*. These proteins show strict hierarchy in structure, being that the interaction of various ligands, such as O_2, CO and NO, and the principle binding behavior of these protein complexes has been considered the main topics to the understanding of the respective structure-function relationship (Hellmann et al., 2008).

12. Giant extracellular hemoglobin of *glossoscolex paulistus*

The hemoglobin (Hb) of the annelid *Glossoscolex paulistus* is a giant extracellular Hb (erythrocruorin) that dissociates into low affinity subunits after incubation under pressure (with stabilization of the dissociated products), alkalization (mainly around pH 9.0) of acidification of medium or surfactant addition (Bispo et al., 2007).

13. The influence of ions on the structure-activity relationship: A comparative evaluation between vertebrate and invertebrate hemoproteins

Elucidation of the detailed thermodynamics of heme site ligation and its linkages to heterotropic effectors has required circumvention of the following obstacles: (i) lability of the heme iron-oxygen bond precludes the isolation and study of most intermediates; (ii) high binding cooperativity greatly reduces the populations of intermediates in equilibrium

with the end-state species; and (iii) dissociation of tetramers into dimmers leads to their reassembly into tetramers with rearranged configurations of occupied sites (Huang et al., 1997). The oxygen binding properties of extracellular giant hemoglobins (Hbs) in some annelids exhibit features significantly different from those of vertebrate tetrameric Hbs. Annelid giant Hbs show cooperative oxygen binding properties in the presence of inorganic cations, while the cooperativities of vertebrate Hbs are enhanced by small organic anions or chloride ions (Numoto et al., 2008). This interesting difference must be associated to several aspects of the distinct structure-function relationship found in vertebrate and invertebrate hemoglobins, respectively. Giant extracellular hemoglobins are known by their acid isoelectric points (pI), which is deeply related to several oligomeric and physico-chemical properties that are peculiar to this hemoprotein systems, when compared with mammalian hemoproteins. In contrast to mammalian or vertebrate tetrameric Hb, invertebrate Hbs show remarkable varieties in terms of quaternary structure and oxygen binding properties. Two types of extracellular giant Hbs occur in some annelids. Earthworm *Lumbricus terrestris* has a 3600 kDa Hb designated as hexagonal bilayer (HBL) Hb, which also occurs in many other annelids. *Lumbricus* Hb shows moderate oxygen affinity and highly cooperative oxygen binding properties coupled with inorganic cations and protons (Numoto et al., 2008). The heterotropic interactions involving inorganic cations are commonly observed features among annelid HBL Hbs. Cations and protons preferably bind to the R state and increase the ligand affinity of HBL Hbs; the heterotropic effectors in the annelid HBL Hbs differ markedly from those of vertebrate Hbs. Another giant Hb from an annelid is a 400 kDa Hb that occurs in some siboglinid polychaetes. *Oligobrachia mashikoi*, a frenulate beard worm, has a 400 kDa Hb composed of four globin subunits (A1, A2, B1, and B2) that form a 24-mer hollow-spherical structure. The oxygen binding properties of *Oligobrachia* Hb are qualitatively similar to those of annelid HBL Hbs. It is important to notice that both the oxygen affinity and cooperativity of *Oligobrachia* Hb are enhanced by the addition of Ca2+ and/or Mg2+, or by an increase in pH (Numoto et al.,, 2008). Oxygenation properties of hemoglobin (Hb) from Oligobrachia mashikoi were extensively investigated. Compared to human Hb, Oligobrachia Hb showed a high oxygen affinity (P50 = 1.4 mmHg), low cooperativity (n = 1.4), and a small Bohr effect (dH+ =_0.28) at pH 7.4 in the presence of minimum salts (Aki, et al., 2007). Addition of NaCl caused no change in the oxygenation properties of Oligobrachia Hb, indicating that Na^+ and Cl^- had no effect. Mg^{2+} and Ca^{2+} remarkably increased the oxygen affinity and cooperativity. Thus, unlike the vertebrate Hbs, but like the annelid extracellular Hbs, the oxygen binding properties of Oligobrachia Hb are regulated by divalent cations which preferentially bind to the oxy form (Aki, et al., 2007).

14. Why the intensity of polypeptide compaction is decisive to the physico-chemical properties of HbGp?

The oligomeric compaction, which is dependent of the level of polypeptide compressibility, is a fundamental aspect of the structure-activity relationship of HbGp. Probably this fact occurs as consequence of two factors: The extraordinarily high hydrophobicity of the compacted arrangement, i.e., the native configuration, and the high restriction to the accessibility of ions, which can induce the occurrence of autoxidation mechanisms.The dielectric constant of the heme pocket neighborhood affects pronouncedly the hydrogen bonds developed by the aminoacid residues around the heme, including the distal and proximal histidines. These two histidines are extraordinarly important in several processes

that control the structure-function of hemoglobins, such as the ligand exchange on the first coordination sphere of the metallic center. The physico-chemical properties of these important residues can be intensely altered when the polarity of medium is modified, which can affect the influence of these residues upon the heme reactivity.

15. The peculiarities of the unfolding mechanism in alkaline and acid media

Venkatesh and co-workers (Venkatesh, 1999), studying hemoglobins reconstituted with Cu-porphyrin and Ni-porphyrin as function of pH, provide significant insight about the mechanism of conservation of the native configuration in hemoproteins. In any case, the work of these authors supports that the alkaline medium propitiates a higher complexity in the equilibria of species when compared with the acid medium (Venkatesh, et al., 1999). The higher complexity of species in alkaline medium when compared with the acid conditions would be associated to some factors. Firstly, the several specific disprotonation processes, which can occur with the different aminoacid residues in alkaline medium, offers a great number of potential ligands to the metallic center, together with the aqueous solvent molecules, which can to coordinate the metallic center as well as its disprotonated form, i.e., the hydroxyl ion.

In acid medium, the potential ligands can be protonated, depending of their respective isoelectric points (pI). This fact limits the number and the efficacy as ligands of the aminoacid residues.An interesting example of the influence of the protonation state on the properties as ligands can be encountered in the evaluation of the histidine as ligand. Actually, histidine is very important ligand to heme proteins, mainly hemoglobins, where can to form the bis-histidine complexes, which are commonly called “hemichromes”. In alkaline conditions, some configuration can be considered effective ligands to the metallic center, while in acidic medium, the number of active states as ligands is pronouncedly lower. In this context, it is relevant to notice that the extraordinary level of compaction of the polypeptide chains probably is related to an intense and well organized interaction between opposite charges. The slight and gradual loss of intra- and inter-chains contacts lightly initiates a process of discompaction that is very difficult to be reversed, mainly in giant extracellular hemoglobins as function of the extraordinarily large supramolecular mass of this class of hemoproteins (approximately 3.6 MDa to *Lumbricus terrestris* and *Glossoscolex paulistus* hemoglobins). In our previous article, which is focused on drastic pH transitions of heme species, it is possible to infer that HbGp presents high level of irreversibility in more drastic pH changes.

16. What are the predominant ferric heme configurations in HbGp?

Bis-imidazole and bis-pyridine complexes of Fe(III) porphyrins,1-3 including the octaalkyltetraphenylporphyrins provide excellent models for bis-histidine coordinated heme centers involved in a number of cytochrome-containing systems, examples of which include cytochromes *b* of mitochondrial Complexes II5 and III6-20 and of chloroplast cytochrome *b6f* (Yatsunyk et al., 2006). In fact, these model complexes, which allow a study focused on the first coordirnation sphere as well as the hemoproteins, such as HbGp, that present more global information, demonstrates that aquomet, hemichrome and pentacoordinate (mono-histidine) species are the predominant forms in heme systems. The tendency of hemichrome hexacoordinated species formation immediately after light

medium perturbation in the neighborhood of the protein suggests a deeply high sensitivity of the F helix, permitting a significant approach of the distal histidine in relation to the metallic center. Nevertheless, after this slight spatial modification, the predominant part of the polypeptide chains tends to lose great number of intra- and inter-chains subunits contacts, favoring a labilization process of the two helices bounded axially to the metallic center through the distance increase between the helices that constitute the secondary protein structure. This mechanical influence compromises the stability of these axial ligations of the metallic center, favoring the respective breakage of one of them, with consequent appearance of a significant presence of pentacoordinated ferric species. Obviously, in a very large supramolecular system, including 180 biological macromolecules, which are the polypeptide chains, depending of the unfolding mechanism, the tension on the axial helices would be significantly different to each subunit, which can justify the coexistence of several heme species. In other words, we can propose that, in spite of some general properties, the polypeptide chains are not submitted strictly to the same unfolding process. This fact can explain the coexistence of distinct ferric heme species in each pH value, mainly when the medium conditions are very distant of the neutrality (pH=7.0).

17. The interaction between HbGp and surfactants as resource of physico-chemical informations

Surfactants are surface-active agent and belong to the category of amphiphilic molecules. They are capable of forming aggregates known as micelles and the concentration at which they form is known as the critical micellar concentration (cmc). Surfactants are widely employed in biochemistry and biotechnology for the purpose of protein solubilization, purification, characterization, and protein structure determination (Miksovska, et al., 2006). Surfactant-protein interactions are very common in the fields of medicine, chemistry, biology and so on (Liu et al., 2007; van der Veen et al., 2004; Orioni et al., 2006; Vasilescu et al., 1999; Stenstam et al., 2003). Many approaches have been focused such as the kind of protein-surfactant interaction, the influence of the aggregation state of the surfactant (monomer, pre-micellar aggregate and micelle) on the protein structure, the properties of the surfactant-protein system, the characterization of the interaction sites on the protein surface, the identification of the intermediate protein conformations, etc (Liu et al., 2007; Liu et al., 2005; Ajloo et al., 2002). Research involving interaction of cationic and anionic surfactants with macromolecules, especially proteins, have been developed (Maulik et al., 1998; Tofani et al., 2004), being the interaction of charged headgroups the main focus of these works. Furthermore, depending of the concentration, surfactants can act as unfolding and denaturant agents, which can be used to evaluate the structural properties of the different proteins and their active sites. Studies of the interaction of globular proteins with surfactants, in particular with sodium dodecyl sulphate (SDS), have been carried on with the aim to understand details of the structure and function of proteins (Yang et al., 2003; Tanford et al., 1973; Gebicka& Gebick, 1999; Chattopadhyay & Mazumbar, 2003). Three ranges of surfactant concentrations associated to different effects on the protein should be mentioned. The first one, at stoichiometric surfactant concentration, is related to the finding that occurs to specific sites on the protein, due to, mainly, electrostatic interactions (Dayer et al., 2002; Moosavi-Movahedi, 2003 et al., Decker et al., 2001). At higher surfactant concentrations, near to the surfactant critical micellar concentration (cmc), where pre-micellar aggregates are formed and a massive increase in protein binding due to cooperative

ligand interactions takes place (Turro, 1995 et al., Jones, 1995). Above the cmc, at milimolar surfactant concentrations, the protein-surfactant interaction is an extremely complicated phenomenon.

Generally, surfactants induce a decrease of the α-helix content, and this effect is smaller for cationic than for anionic ones (Tofani et al., 2004). However, in the case of the giant extracellular hemoglobins, which are also called erytrocruorins, such as *Glossoscolex paulistus* hemoglobin, this is not true (Santiago et al., 2007). Probably, this is associated to the decisive influence of the value of isoelectric point (pI) in the control of the interaction with ionic surfactants, since the erytrocruorins are proteins with very acid pI, differently of the most of the hemoproteins. Otzen and Oliveberg (Otzen et al., 2002), studying a small protein S6 in the presence of SDS, argue that monomeric SDS binds to the native state, but global unfolding would occur only above the critical micelle concentration (cmc). Indeed, this verification is corroborated for various works about interaction between surfactants and hemoproteins (Oellerich et al., 2003; Tofani et al., 2004; Das et al., 1999). Oellerich and co-workers (Oellerich et al., 2003), analyzing the interaction between SDS and cytochrome *c*, explain that the differences observed below and above the cmc are due to the different modes of binding of SDS monomers and micelles. These authors argue that alterations of the heme structure are common to both modes of interaction, implying that the sites of electrostatic and hydrophobic contacts should be located in the vicinity of the cytochrome c heme pocket. In agreement with this discussion, Tofani and co-workers (Tofani et al., 2004) observed for Horse myoglobin (MbH), that only in a SDS/MbH ratio higher than 400, would occur a more significant protein unfolding and, for consequence, a more exposed and accessible heme pocket. Studies focused on the SDS-cytochrome *c* interaction at neutral pH have demonstrated that the unfolded state is stabilized when occur the bis-histidine (hemichrome) species formation, being that subsequent modifications of the secondary structure are rate-limited by the histidine dissociation rate (Das et al., 1999). Therefore, the hemichrome formation anchors the E helix into the ferric center, and, with the E and F helices maintained connected to the metallic center, the new polypeptide chains arrangement is stabilized, at least partially. This observation illustrates the correlation between the oligomeric assembly changes with the modifications that occur in the first coordination sphere of the metal in hemoproteins.

The dependence of the kind of protein-surfactant interaction with the surfactant concentration is also found to other types of protein. Bovine Serum Albumin (BSA), for example, presents initial high affinity binding sites for surfactants, which correspond to an intense electrostatic binding, characteristic for anionic compounds and BSA, at higher surfactant concentrations become some sort of nucleation sites for binding of "micelle-like" aggregates (Gelamo et al., 2004). In spite of the great predominance of the electrostatic contribution in the interaction of SDS with hemoproteins, the hydrophobic contacts present a significant importance to the total interaction. Gebicka and co-workers (Gebicka & Gebicki, 1999) argue that the hydrocarbon part geometry from the surfactant in the SDS-cytochrome *c* interaction is responsible for great influence on the spatial configuration of the heme pocket. Polar lipids of biological membranes are predominantly zwitterionic phophatidylcholine and phophatidyllethanolamine. Therefore, micelles of the zwitterionic surfactant N-hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (HPS) may be considered as a convenient model of lipid aggregates (Yushmanov et al., 1994). In spite of this significant biological relevance, the number of works in the literature focusing on the interaction of zwitterionic surfactants, such as HPS, with hemeproteins is significantly lower

as compared to the studies regarding the interactions of hemeproteins and cationic and anionic surfactants.

18. Technological applications of hemoproteins

The understanding of interactions between proteins and surfaces is critically important in many fields of biochemical science, from biosensors to biocompatible materials. The development of biosensors, for instance, presents a critical step that is the immobilization of proteins with intact functions onto the surface of a transducer. Although many proteins spontaneously adsorb onto solid surfaces, the adsorbed proteins often denature or adapt undesirable orientation on the surfaces. In order to avoid these problems, various methods have been developed, each with its own advantages and disadvantages (Boussaad & Tao, 1999).

In recent years, a novel technique of layer-by-layer self-assembly has aroused more interests among researchers and has been developed into a general approach for fabricating ultrathin films on solid surfaces. This molecular architecture method is fundamentally based on the alternate adsorption of oppositely charged species from their solutions with precise thickness control on the nanometer scale. The layer-by-layer assembly has been extended to building up protein or other biomacromolecules films and has been successfully employed for the design and construction of biodevices (Shen & Hu, 2005). It is important to notice, for example, the development of a novel method for fabricating hydrogen peroxide (H_2O_2) sensor, which has been presented based on the self-assembly of ZrO_2 nanoparticles with heme proteins on functional glassy carbon electrode (Zhao et al., 2005). The immobilized proteins performed their direct electrochemistry with the formal potential of -0.032 V for hemoglobin and -0.026 V for myoglobin in pH 6.0, respectively. In fact, the resultant heme-protein electrode exhibited fast amperometric response (within 10 seconds) to H_2O_2, excellent stability, long-term life (more than one month) and good reproducibility (Zhao et al., 2005). Another interesting study was elaborated by Yao and co-workers (Yao et al., 2006), which made a electrochemical and electrocatalytic system of hemoglobin immobilized on glass carbon electrode containing gelatine films that displayed a fast amperometric response to the reduction of H_2O_2 and nitrite (Yao et al., 2006).

19. Hemoproteins as oxygen carrier substitutes and biossensors: Future perspectives

The infusion of Hb-based O_2 carriers (HBOCs) generally causes vasoconstriction and hypertension due to their ability to efficiently scavenge endothelial-derived nitric oxide (NO) and attenuates the vasodilatory NO signal to the vascular smooth muscle (Olson et al., 2004; Cole et al., 2009). However, great efforts are being developed in order to avoid or, at least, attenuate this limitation, altering some variables associated to the application of the hemoproteins as artificial blood. Indeed, the increasing perspectives of this area are motivating several research groups to test the giant extracellular hemoglobins due to its autoxidation resistance and immunological suitability. The applications as biosensor are also interesting employments of this class of hemoglobin due to great affinity to several ligands with great biological relevance, such as NO, CO, O_2, CN-, and several aminoacids and peptides. This kind of iron ligands constitutes a very important group to the physiological medium of several various species. Moreover, these ligands are relevant

chemical species to several types of environmental conditions, implying that the giant extracellular hemoglobins, such as HbGp, could be applied as biosensor in physiological and environmental media.

20. Technological applications of surfactant-hemoprotein systems

Several applications have been developed employing the interactions between proteins and surfactants (Shan et al., 2008; Hu et al., 2007). An interesting work was developed by Shan and co-workers (Shan et al., 2008), which elaborated a self-assembled electroactive layer-by-layer film of heme proteins with anionic surfactant dihexadecyl phosphate. This film grown on pyrolytic graphite (PG) electrodes, showing a pair of well-defined and nearly reversible cyclic voltammetry peaks at around -0.35 V vs SCE at pH 7.0, which is characteristic of the heme protein Fe(III)/Fe(II) redox couples. Hu and co-workers (Hu, 2007) developed similar film using the cationic surfactant didodecyldimethylammonium bromide (DDAB) on PG electrodes and demonstrated two pairs of nearly reversible redox peaks at approximately -0.22 and -1.14 V vs SCE at pH 7.0, which are typical of the hemoglobin Fe(III)/Fe(II) and Fe(II)/Fe(I) redox couples, respectively. Based on the direct electrochemistry of heme proteins, the films could also be applied to electrochemically catalyze reduction of oxygen, hydrogen peroxide and nitrite with significant lowering of reduction overpotentials.

21. Conclusions

The hemoglobin-surfactant interactions also produces oxidation of the metallic center, originally in the ferrous form, into the ferric state. The complex oligomeric assembly of HbGp subunits may influence the autoxidation rate and the exponential decay behavior. In fact, a synergic process in the sequence dissociation-oxidation-dissociation takes place, which means that an initial dissociation favors the occurrence of a more intense oxidation that, in its time, favors a secondary dissociation process. Subsequently, this synergic cycle continues uninterruptedly until a substantial dissociation of the protein fractions, characterizing a kind of oxidation-dissociation cooperative effect. This point is very interesting, since the hemes present a very similar behavior in terms of ligand affinity and spectroscopic profile to the native form of HbGp, in spite of their significant structural differences. This distinction can be observed in the recent studies employing MALDI-TOF-MS, which denote the great number of coexistent isoforms in each subunit (Oliveira et al., 2007; Oliveira et al., 2008). In fact, the oligomeric compaction of the native form works as a kind of equalization of the different hemes. When the dielectric constant of the medium increases as function of the higher freedom of the polypeptide chains, which provokes higher aqueous solvent accessibility into heme pocket, the kinetic behavior of the chains becomes significantly altered. In this way, it is possible to infer that the global environment around the hemes is useful to preclude the iron oxidation as well as to equalize the heme properties. Indeed, an extraordinarily complex arrangement with 180 polypeptide chains represents effective shielding to all hemes of the integral HbGp. This analysis would explain the extraordinarily simple and "pure" RPE spectrum obtained at pH 7.0 with HbGp in a native state, in spite of the evident difference between the heme groups, which is demonstrated when the aqueous solvent accessibility into heme pocket is intensified. These data are in agreement with Moreira and co-workers (Moreira et al., 2006), which demonstrate representative presence of aquomet species until pH 4.0. This ferric species is

very sensitive to perturbations in the heme pocket neighborhood, being very unstable in alkaline medium (Moreira et al., 2008), which reinforces the higher oligomeric stability of HbGp in acid medium when compared with the basic environment. It is important to register that the mono-aquo ferric pentacoordinate species can be encountered into the heme pocket or free in the aqueous solvent, being very difficult to differentiate both pentacoordinate species as function of their similarities with respect to their spectroscopic data. In fact, the ferric porphyrins, which is free in water solvent maintains its pentacoordinated configurations, i.e., the number of coordination continues being five (5). This occurs due to the electronic configuration of metallic centers d^5, which usually are more stable when compared with tetracoordinated configurations (number of coordination four (4). Therefore, it is plausible to infer that the compaction level of the whole HbGp is much more conserved in acid medium, when compared with alkaline one. Furthermore, the level of assembly of the globin chains must be responsible to the difficult of heme distinction, when the native state of this protein is well conserved. The present article demonstrates that the high level of oligomeric compaction is a determinant factor to the "equalization" of the 180 hemes of HbGp. In fact, in spite of their different properties when dissociated, the hemes present very similarity in the native state of HbGp at pH 7.0. However, the interaction with small concentration of ionic surfactants (CTAC and SDS) generates a significant increase in the size of the supramolecular system, denoting a representative loss of oligomeric compaction. Indeed, this original fact is comprehensible in a protein with an extraordinary molecular mass around 3.6 MDa, such as HbGp. This point is decisive to the structure-function relationship to HbGp, and probably to others giant extracellular hemoglobins, since is associated to several relevant phenomenons, such as ligand exchange, oligomeric dissociation and autoxidation. In addition, our present results demonstrate the importance of the isoelectric point in the modulation of protein-surfactant interactions, especially the electrostatic contribution at low surfactant concentrations, suggesting that this parameter is decisive to determine the oligomeric arrangement, as well as the structure-activity relationships.

22. References

Aki, Y., Nakagawa, T., Nagai, M., Sasayama, Y., Fukumori, Y. & Imai, K. (2007). Oxigenation properties of extracellular giant hemoglobin from *Oligobrachia mashikoi*. *Biochemical and Biophysical Research Communications*, Vol. 360, pp. 673-678.

Arndt, M.H.L. & Santoro, M.M. (1998). Structure of the extracellular hemoglobin of *Biomphalaria glabrata*. *Comparative Biochemistry and Physiology B,* Vol. 119, pp. 667-675.

Arndt, M.H.L., de Oliveira, C.L.P. &, Regis, W.C.B. (2003). Torriani IL, Santoro MM, Small angle X-ray scattering of the hemoglobin from *Biomphalaria glabrata*. *Biopolymers*, Vol. 69, PP. 470-479.

Berry, E.A. & Walker, F.A. (2008). Bis-histidine-coordinated hemes in four-helix bundles:how the geometry of the bundle controls the axial imidazole plane orientations in transmembrane cytochromes of mitochondrial Complexes II and III and related proteins. *Journal of Biological Inorganic Chemistry*, Vol.13, pp.481-498.

Bispo, J.A.C., Landini, G.F., Santos, J.L.R., Norberto, D.R. & Bonafe, C.F.S. (2005). Tendency for oxidation of annelid hemoglobin at alkaline pH and dissociated

states probed by redox titration. *Comparative Biochemistry and Physiology B*, Vol. 141, pp. 498-504.

Bispo, J.A.C., Santos, J.L.R., Landini, G.F., Gonçalves & J.M., Bonafe, C.F.S. (2007). pH dependence of the dissociation of multimeric hemoglobin probed by high hydrostatic pressure. *Biophysical Chemistry*, Vol. 125, pp. 341-349.

Boussaad, S. & Tao, N.J. (1999). Electron Transfer and Adsorption of Myoglobin on Self-Assembled Surfactant Films: An Electrochemical Tapping-Mode AFM study. *Journal of the American Chemical Society,* Vol. 121, pp. 4510-4515.

Bruneaux, M., Rousselot, M., Leize, E., Lallier, F.H. & Zal, F. (2008). The structural analysis of large noncovalent oxygen binding proteins by MALLS and ESI-MS: A review on annelid hexagonal bilayer hemoglobin and crustacean hemocyanin. *Current Protein & Peptide Science*, Vol. 9, pp. 150-180.

Chattopadhyay, K. & Mazumbar, S. (2003). Stabilization of partially folded states of cytochrome C in aqueous surfactant: effects of ionic and hydrophobic interactions. *Biochemistry,* Vol. 42, pp. 14606-14613.

Cole, R.H., Malavalli, A. & Vandegriff, K.D. (2009). Erythrocytic ATP release in the presence of modified cell-free hemoglobin. *Biophysical Chemistry*, Vol. 144, pp.119-122.

Das, T. P., Boffi, A., Chiancone, E. & Rousseau, D. L. (1999). Hydroxide Rather Than Histidine Is Coordinated to the Heme in Five-coordinate Ferric *Scapharca inaequivalvis*Hemoglobin *J. Biol. Chem*. 1999, 274, 2916.

Dayer, M.R. ,Moosavi-Movahedi, A.A., Norouzi, P., Ghourchian, H.O. & Safarian, S. (2002). Inhibition of human hemoglobin autoxidation by sodium n-dodecyl sulphate. *Journal of Biochemical and Molecular Biology,* Vol. 35, pp.364-370.

Decker, H., Ryan, M., Jaenicke, E. & Terwilliger, N. (2001). SDS-induced phenoloxidase activity of hemocyanins from *Limulus polyphemus, Eurypelma californicum,* and *Cancer magister*. *The Journal of Biological Chemistry*, Vol. 276, pp. 17796-17799.

Feng, Y., Roder, H. & Englander, S.W. (1990) Redox-Dependent Structure Change and Hyperfine Nuclear Magnetic Resonance Shifts in Cytochrome *c*. *Biochemistry* , Vol. 29, pp. 3494-3504.

Gebicka, L. & Gebicki, J.L. (1999). Kinetic studies on the interaction of ferricytochrome C with anionic surfactants. *Journal of Protein Chemistry*, Vol. 18, pp.165-172.

Gelamo, E. L., Itri, R. & Tabak, M. (2004). Small Angle X-Ray Scattering (SAXS) Study of the Extracellular Hemoglobin of Glossoscolex paulistus: Effect of pH, Iron Oxidation State and Interaction With Anionic SDS Surfactant. *The Journal of Biological Chemistry,* Vol. 279, pp. 33298-33305.

Gell, D.A., Feng, L., Zhou, S., Jeffrey, P.D., Bendak, K., Gow, A., Weiss, M.J., Shi, Y. & Mackay, J.P. (2009). A *cis*-Proline in Hemoglobin Stabilizing Protein Directs the Structural Reorganization of Hemoglobin**.** *The Journal of Biological Chemistry,*Vol. 284, pp. 29462-29469.

Harrington, J.P., Kobayashi, S., Dorman, S.C., Zito, S.L., & Hirsch, R.E. (2007). Acellular invertebrate hemoglobins as model therapeutic oxygen carriers: Unique redox potentials. *Artificial Cells Blood Substitutes and Biotechnology* , Vol. 35, pp. 53-67.

Hellmann, N., Weber, R.E. & Decker, H. (2008). Linker analysis of large cooperative, allosteric systems: The case of the giant HBL hemoglobins, Globins and other nitric oxide-reactive proteins. *Methods in Enzymology*, Vol. 436, pp. 436-485.

Hocking, R.K., Wasinger, E.C., Yan, Y., deGroot, F.M.F., Walker, F.A. Hodgson, K.O., Hedman, B. & Solomon, E.I. (2007). Fe L-Edge X-ray Absorption Spectroscopy of Low-Spin Heme Relative to Non-heme Fe Complexes: Delocalization of Fe d-Electrons into the Porphyrin Ligand, *Journal of the American Chemical Society,* Vol.129, pp.113-125.

Hocking, R.K., Wasinger, E.C., Yan, Y.L., deGroot, F.M.F., Walker, F.A., Hodgson, K.O. & Hedman, B. (2007). Solomon EI, Fe L-edge x-ray absorption spectroscopy of low-spin heme relative to non-heme Fe complexes: Delocalization of Fe d-electrons into the porphyrin ligand. *Journal of the American Chemical Society*, Vol. 129, pp.113-125.

Hu, Y., Sun, H. & Hu, N. (2007). Assembly of layer-by-layer films of electroactive hemoglobin and surfactant didodecyldimethylammonium bromide. *Journal of Colloid and Interface Science,* Vol. 314, pp. 131-140.

Huang, Y.W., Koestner, M.L. & Ackers, G.K. (1997). Tertiary and quaternary chloride effects of the partially ligated (CN-met) hemoglobin intermediates. *Biophysical Chemistry*, Vol. 64, pp. 157-173.

Jones, M.N. (1992). Surfactant interactions with biomembranes and proteins. *Chemical Society Reviews*, Vol. 21, pp. 127-136.

Knipp, K., Yang, F., Berry, R.E., Zhang, H., Shokhirev, M.N. & Walker, F.A. (2007). Spectroscopic and Functional Characterization of Nitrophorin 7 from the Blood-Feeding Insect *Rhodnius prolixus* Reveals an Important Role of Its Isoform-Specific N-Terminus for Proper Protein Function. *Biochemistry*, Vol. 46, pp. 13254-13268.

Kolczak, U., Han, C. Sylvia, L.A. & La Mar, G.N. (1997). Approaches to the Solution NMR Characterization of Active Sites for 65 kDa Tetrameric Hemoglobins in the Paramagnetic Cyanomet State. *Journal of the American Chemical Society,* Vol. 119, pp. 12643-12654.

Liu, G., Shao, W., Huang, X., Wu, H. & Tang, W. (1996). Structural studies of imidazole-cytochrome *c*: resonance assignments and structural comparison with cytochrome *c*, *Biochimica et Biophysica Acta*, Vol. 1277, pp. 61-82.

Liu, W., Guo, X. & Guo, R. (2007). The Interaction of Hemoglobin with two Surfactants of Different Charges, *International Journal of Biological Macromolecules*, Vol. 41, pp. 548-557.

Maulik, S., Dutta, P., Chattoraj, D.K. & Moulik, S.P. (1998). Biopolymer-surfactant interactions: 5 Equilibrium studies on the binding of cethyltrimethyl ammonium bromide and sodium dodecyl sulfate with bovine serum albumin, β-lactoglobulin, hemoglobin, gelatin, lysozyme and deoxyribonucleic acid. *Colloids and Surfaces B,* Vol.11, pp. 1-8.

Miksovska, J., Yom, J., Diamond, B. & Larsen, R.W. (2006). Spectroscopic and Phototermal Study of Myoglobin Conformational Changes in the Presence of Sodium Dodecyl Sulfate. *Biomacromolecules*, Vol. 7, pp.476-482.

Moosavi-Movahedi, A.A., Dayer, M.R., Norouzi, P., Shamsipur, M., Yeganeh-faal, A., Chaichi, M.J. & Ghourchian, H.O. (2003). Aquamethemoglobin reduction by

sodium n-dodecyl sulfate via coordinated water oxidation. *Colloids and Surfaces B*, Vol. 30, pp. 139-146.

Moreira, L. M., Lima Poli, A., Costa Filho, A. & Imasato, H. (2006). Pentacoordinate and hexacoordinate ferric hemes in acid medium: EPR, UV Vis and CD studies of the giant extracellular hemoglobin of Glossoscolex paulistus. *Biophysical Chemistry*, Vol. 124, pp. 62-72.

Moreira, L., Poli, A., Costa Filho, A. & Imasato, H. (2008). Ferric species equilibrium of the giant extracellular hemoglobin of Glossoscolex paulistus in alkaline medium: HALS hemichrome as a precursor of pentacoordinate species. *International Journal of Biological Macromolecules*, Vol. 42, pp. 103-110.

Numoto, N., Nakagawa, T., Kita, A., Sasayama, Y., Fukumori, Y. & Miki, K. (2008). Structural Basis for Heterotropic and Homotropic Interactions of Invertebrate Giant Hemoglobin. *Biochemistry*, Vol. 47, pp.11231-11238.

Oliveira, M., Moreira, L. & Tabak, M. (2007). Partial characterization of giant extracellular hemoglobin of Glossoscolex paulistus: A MALDI-TOF-MS study. *International Journal of Biological Macromolecules*, Vol. 40, pp. 429-436.

Oliveira, M., Moreira, L., Tabak, M. (2008). Interaction of giant extracellular Glossoscolex paulistus hemoglobin (HbGp) with ionic surfactants: A MALDI-TOF-MS study. *International Journal of Biological Macromolecules*, Vol. 42, pp. 111-119, 2008

Olson, J.S., Foley, E.W., Rogge, C., Tsai, A.L., Doyle, M.P. & Lemon, D.D. (2004). NO scavenging and the hypertensive effect of hemoglobin-based blood substitutes. *Free Radical in1 Biological Medicine*, Vol. 36, pp. 685–697.

Orioni, B., Roversi, M., La Mesa, C., Asaro, C., Asaro, F., Pellicer, G. & D´Errico, G. (2006). Polymorphic behavior in protein-surfactant mixtures: the water-bovine serum albumin-sodium taurodeoxycholate system. *Journal of Physical Chemistry B,* Vol 110, pp.12129-12140.

Pereira, M.T., Silva-Alves, J.M., Martins-José, A., Lopes, J.C.D. & Santoro, M.M. (2005). Thermodynamic evaluation and modeling of proton and water exchange associated with benzamidine and berenil binding to ß-trypsin. *Brazilian Journal of Medical and Biological Research,* Vol. 38, pp. 1593-1601.

Royer, W.E., Strand, K. M. & Hendrickson, W.A. (2000). Structural hierarchy in erythrocruorin, the giant respiratory assemblage of annelids, *PNAS*, Vol. 97, pp. 7101-7111.

Sakudo, A., Kato, Y.H., Kuratsune, H. & Ikuta, K. (2009). Non-invasive prediction of hematocrit levels by portable visible and near-infrared spectrophotometer. *Clinica Chimica Acta,* Vol. 408, pp. 123-127.

Santiago, P.S., Moreira, L. M., de Almeida, E.V. & Tabak, M. (2007). Giant extracellular *Glossoscolex paulistus* Hemoglobin (HbGp) upon interaction with cethyltrimethylammonium chloride (CTAC) and sodium dodecyl sulphate (SDS) surfactants: Dissociation of oligomeric structure and autoxidation. *Biochimica et Biophysica Acta. G, General Subjects* (Print), vol. 1770, pp. 506-517.

Shan, W., Liu, H., Shi, J., Yang, L. & Hu, N. (2008). Self-assembly of electroactive layer-by-layer films of heme proteins with anionic surfactant dihexadecyl phosphate. *Biophysical Chemistry,* Vol. 134, pp. 101-109.

Shao, W. , Liu, G., Huang, X. , Wu, H. & Tang, W. (1996) 2D-NMR studies of the effects of axial substitution on two helices in horse cytochrome *c*, *Biochimica et Biophysica Acta,*Vol. 1295, pp. 44-50.

Shen, L. & Hu, L.N. (2005). Electrostatic Adsorption of Heme proteins Alternated with Polyamidoamine Dendrimers for Layer-by-Layer Assembly of Electroactive Films. *Biomacromolecules,* Vol. 6, pp.1475-1483.

Stenstam, A., Montalvo, G., Grillo, I. & Gradzielski, M. (2003). A sans study of lysozyme-sds aggregates, *Journal of Physical Chemistry B,* Vol. 107, pp.12331-12338.

Tanford, C. The Hydrophobic Effect: Formation of Micelles and Biological Membranes, Wiley, New York, 1973.

Tofani, L., Feis, A., Snoke, R.E., Berti, D., Baglioni, P. & Smulevich, G. (2004). Spectroscopic and interfacial properties of myoglobin/surfactant. *Biophysical Journal*, Vol. 87, pp. 1186-1195.

Turro, N.J., Lei, X.G., Ananthapadmanabhan, K.P. & Aronson, M. (1995). Spectroscopic probe analysis of protein-surfactant interactions – The BSA/SDS System. *Langmuir*, Vol. 11, pp. 2525-2533.

van der Veen, M., Norde, W. & Stuart, M.C. (2004). Electrostatic interactions in protein adsorption probed by comparing lysozyme and succinylated lysozyme. *Colloids and Surfaces B,* Vol. 35, pp. 33-40.

Vasilescu, M., Angelescu, D., Alegren, M. & Valstar, A. (1999). Interactions of globular proteins with surfactants studied with fluorescence probe methods . *Langmuir,* Vol.15, pp.2635-2643.

Venkatesh, B., Ramasamy, S., Mylrajan, M., Asokan, R., Manoharan, P.T. & Rifkind, J.M. (1999). Fourier transform Raman approach to structural correlation in hemoglobin derivatives. *Spectrochimica Acta A*, Vol. 55, pp. 1691-1697.

Walker, F.A. (2006). The heme environment of mouse neuroglobin: histidine imidazole plane orientations obtained from solution NMR and EPR spectroscopy as compared with X-ray crystallography. *Journal of Biological Inorganic Chemistry,* Vol. 11, pp. 391-397.

Weber,R.E.& Vinogradov, S.N. (2001). Nonvertebrate Hemoglobins: Functions and Molecular Adaptations, Vol. 81, pp. 569-628.

Yang, X., Guo, X. & Li, H. (2003). Fluorimetric determination of hemoglobin using spiro form rhodamine B hydrozide in a micellar medium. *Talanta,* Vol.61, pp.439-445.

Yao, H., Li, N., Xu, J. & Zhu, J. (2007). Direct electrochemistry and electrocatalysis of hemoglobin in gelatine film modified glassy carbon electrode. *Talanta,* Vol. 71, pp. 550-554.

Yatsunyk, L.A., Dawson, A., Carducci, M.D., Nichol, G.S. & Walker, F.A. (2006). Models of the Cytochromes: Crystal Structures and EPR Spectral Characterization of Low-Spin Bis-Imidazole Complexes of (OETPP)FeIII Having Intermediate Ligand Plane Dihedral Angles. *Inorganic Chemistry,* Vol. 45, pp. 5417-5428.

Yushmanov, V. E., Perussi, J. R., Imasato, H., Ruggiero, A. C. & Tabak, M. (1994). Ionization and binding equilibria of papaverine in ionic micelles studied by 1H-NMR and optical absorption spectroscopy. *Biophysical Chemistry*, Vol. 52, pp. 157-163. Estados Unidos, v. 52, p. 157-163, 1994.

Zhao, G., Feng, J., Xu, J. & Chen, H. (2005). Direct electrochemistry and electrocatalysis of heme proteins immobilized on self-assembled ZrO_2 film. *Electrochemistry Communications,* Vol. 7, pp. 724-729.

Biosensors for Health, Environment and Biosecurity

Edited by Prof. Pier Andrea Serra

ISBN 978-953-307-443-6

Hard cover, 540 pages

Publisher InTech

Published online 19, July, 2011

Published in print edition July, 2011

A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 16 different countries. The book consists of 24 chapters written by 76 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen