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Interaction of *Glossoscolex paulistus* extracellular hemoglobin with hydrogen peroxide: Formation and decay of ferryl-HbGp

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ABSTRACT

The giant extracellular hemoglobin from earthworm *Glossoscolex paulistus* (HbGp) reacts with hydrogen peroxide, displaying peroxidase activity in the presence of guaiacol. The formation of ferryl-HbGp (compound II) from the peroxidase cycle was studied in the present work. The hypervalent ferryl-HbGp species was formed directly by the reaction of oxy-HbGp and hydrogen peroxide. The oxy-HbGp heme groups (144) under different excess of H_2O_2, relative to heme, showed an influence in the total amount of ferryl-HbGp at the end of the reaction. The ferryl-HbGp was formed with second order rate constant of $27.1 \pm 0.5\ M^{-1}\ s^{-1}$, at pH 7.0 and 25 °C. The increase of the pH value to 8.0 induces both faster formation and decay of ferryl-HbGp, together with oligomeric dissociation induced by the presence of H_2O_2, as observed by DLS. This effect of dissociation increases the heme exposure and decreases the ferryl-HbGp stability, affecting the rate constant as a parallel reaction. At pH 7.0, high excess of H_2O_2, above 1:5 oxy-HbGp heme: H_2O_2, produces the aggregation of the protein. Our results show for the first time, for an extracellular giant hemoglobin, the possible effects of oxidative stress induced by hydrogen peroxide.

1. Introduction

Annelids lack the special red blood cells structure, that protect the hemoglobins from oxidative damage [1]. Instead, the hemoglobins from annelids are giant extracellular structures, which are found in the organism hemolymph. Giant extracellular hemoglobin from many species, such as *Lumbricus terrestris* (HbLt) and *Arenicola marina* (HbAm), have been studied with the aim to develop a hemoglobin-based oxygen carrier (HBOC) due to the excellent oxygen binding capacity and oxidative stability in their native extracellular ambient and in vitro solutions. These properties enable longer storage as compared to the human whole blood [1,2]. The structure and properties of the giant hemoglobin of *Glossoscolex paulistus* (HbGp) are very similar to those of HbLt hemoglobin [3]. The 144 oxygen binding sites of the HbGp, 36 times higher than human hemoglobin, provide the stable Iron II oxy- form with low rate of oxidation [4].

HbGp is an extracellular heme protein (hemoglobin), extracted from the earthworm of the same name, with remarkable high redox stability of the oxy-iron heme state. The protein oligomeric structure is composed of twelve protomers constituted by a dodecamer of four globins named ***a***, ***b***, ***c*** and ***d***, $(\boldsymbol{abcd})_3$. The ***abc*** globin chains trimer is maintained by disulfide bonds and the tetramer (***abcd***) is an association of trimer ***abc*** to the monomeric subunit, monomer ***d***. The dodecamer structure is bound to three non-globin linkers $\boldsymbol{L}_i$ ($i = 1$–3), forming the protomer $(\boldsymbol{abcd})_3\boldsymbol{L}_3$. Each globin contains one iron heme group resulting in a total of 144 heme groups in the whole structure [4]. A representation of the oligomeric structure and some details of its constituents based in the reported crystal structure [4] is given in Scheme 1.

The reaction of heme iron with hydrogen peroxide (H_2O_2) has been investigated in proteins and enzymes systems [5–8]. The two electron oxidation of heme iron prosthetic group by hydrogen peroxide results in the formation of perferryl radical and ferryl intermediate species. Ferryl and perferryl species are recognized as triggers of oxidative processes and damage to biomolecules [9,10]. Furthermore, under some conditions the reaction may even result in heme degradation and iron release [11–13]. Hypervalent intermediate half-life and the heme group degradation depend on the oxidation state of iron atom (ferrous or ferric), the excess of the hydrogen peroxide and the presence of a reducing agent [14–15].

The interaction of these heme proteins with the endogenous oxidant such as hydrogen peroxide [8] should induce oxidative stress in the organism. Oxidative damage to the protein structure can also cause the instability (e.g. aggregation) of the oxygen carriers in the blood stream. In a recent study the HbGp peroxidase activity, at pH 7.0, in the presence of guaiacol and H_2O_2 was studied, indicating a significant peroxidase-like antioxidant activity and formation of several oxidized species in the solution. However, the mechanism and the intermediate ferryl- species

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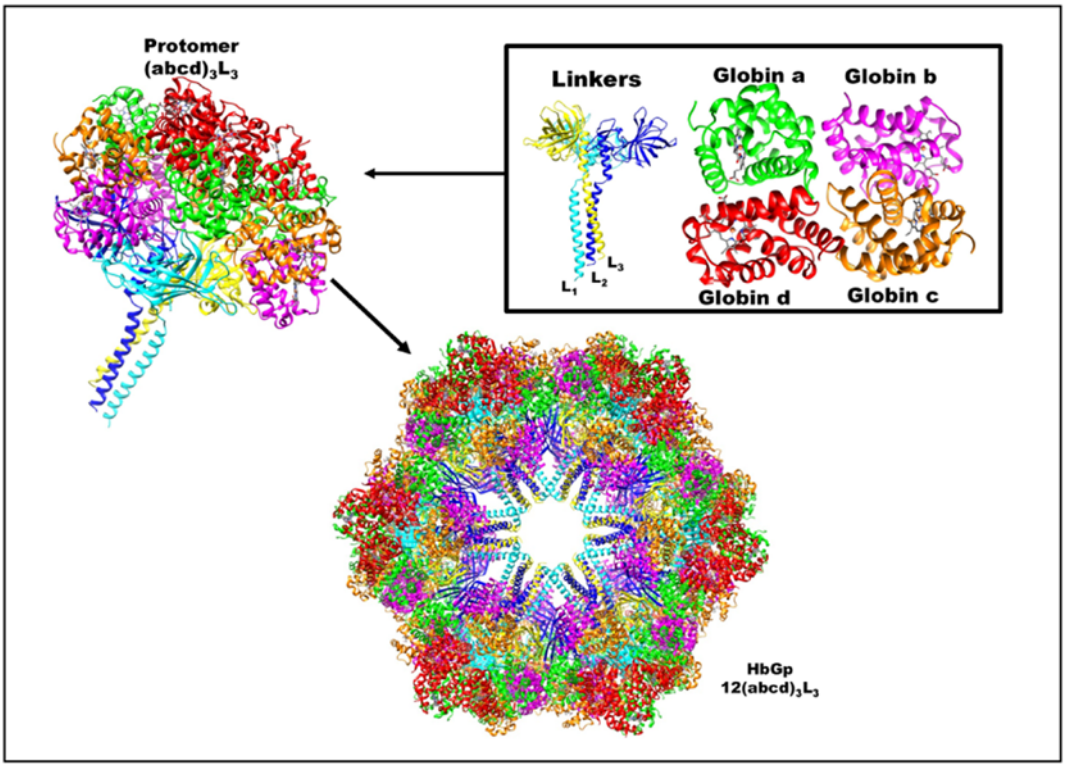

Scheme 1. Representation of the crystal structure of HbGp based on the PDB entry 4U8U [4].

have not yet been investigated in detail for HbGp and the other known extracellular hemoglobins [8]. The reaction of HbGp hemoglobin with hydrogen peroxide should provide a model for the development of basic knowledge about the oxidative damage to the structure of extracellular hemoglobins, and formation of the reactive species which are involved in redox reactions with biological significance.

The present study aims to evaluate, for the first time, the formation of ferryl species from the reaction of extracellular hemoglobin from *Glossoscolex paulistus* with hydrogen peroxide. The results described in the present study, should be used as the basis to explain chemical and structural changes following the interaction of extracellular hemoglobins with biological oxidants. They also may contribute in the evaluation of the uses of extracellular hemoglobins as blood substitutes, as well as, in their storage.

2. Material and methods

2.1. Purification and preparation of oxy-HbGp and met-HbGp

The hemoglobin was isolated from *Glossoscolex paulistus* fresh blood using the procedure as previously described [16–17]. Fresh blood sample extracted from the animal was centrifuged at 4 °C (2300 ×*g* for 15 min) to remove the cells residues (cellular debris). In order to eliminate the low molecular weight components the ultra-filtration was performed using a molecular mass cut-off of 30 kDa in 0.1 M Tris–HCl buffer pH 7.0, at 4 °C. The protein HbGp was obtained as a pellet after the ultracentrifugation at 250,000 ×*g*, at 4 °C, for 3 h and resuspended using a minimal volume of Tris–HCl 0.1 M buffer, at pH 7.0, and stored in the oxy-HbGp form, at 4 °C. An additional purification using Sephadex G-200 column was performed to obtain the stock solution used in the experiments. The oxidized form met-HbGp was obtained by the reaction of oxy-HbGp with 5-fold excess of potassium ferricyanide relative to heme groups for 2 h. In order to remove low molecular weight compounds an exhaustive dialysis was performed. The final concentration of stock solutions were determined spectrophotometrically using the molar extinction coefficients $\varepsilon_{415nm} = 5.5 \pm 0.8$ $(mg/mL)^{-1}$ cm^{-1} for oxy-HbGp and $\varepsilon_{402\ nm} = 4.1 \pm 0.7$ $(mg/mL)^{-1}$ cm^{-1} for met-HbGp [18–21].

2.2. Kinetic experiments

Stock solutions of HbGp with final concentrations of 0.1 (to monitor the whole spectra) and 0.6 mg/mL (to monitor the isolated Q-bands spectra) were mixed, in a 1 cm optical path length quartz cell (Hellma GmbH and Co. KG, Müllheim, Germany), with hydrogen peroxide solution, ranging from equimolar (1:1) to 10-fold (1:10) relative to the concentration of heme groups, which is equal to 4 μM (0.1 mg/mL of protein) and 24 μM (0.6 mg/mL of protein). The spectra acquisition started immediately after mixing using Hitachi U2000 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) equipped with a Peltier temperature controller. Kinetic experiments were performed in phosphate buffer 100 mM, at pH values 7.0 or 8.0, and 25 °C.

2.3. Data analysis using Convex Constraint Algorithm (CCA)

The reaction of oxy-HbGp (Fe^{2+}) with H_2O_2 follows the mechanism of direct two electrons oxidation of iron by H_2O_2 resulting in the ferryl-oxo species ($Fe^{4+} = O$). However, the formation of the ferryl-oxo compound is difficult to be identified from the UV-VIS spectra measurements, especially at low concentrations of H_2O_2, as will be shown later in Section 3.3, due to the superposition of ferryl-HbGp and oxy-HbGp spectra. Furthermore, for longer time periods, a third species, hemichrome, is formed, due to the ferryl-HbGp decay. It presents a spectrum, overlapping the two species mentioned previously (oxy-HbGp and ferryl-HbGp). Hemichrome is an Fe^{3+} porphyrin species where the distal histidine is coordinated to the iron metal, replacing the water molecule at the sixth coordination in the aquomet species [22]. The evolution of the solution species composition with time was monitored by the calculation of the contribution of the pure components (oxy-HbGp, ferryl-HbGp and hemichrome) to the final spectra. These spectral analyzes were performed using the software CCA plus [23–25]. It is based on the Convex Constraint Algorithm (CCA),