International Journal of Biological Macromolecules 92 (2016) 670–681

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Ionic surfactants-*Glossoscolex paulistus* hemoglobin interactions: Characterization of species in the solution

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ARTICLE INFO

Article history:
Received 29 April 2016
Received in revised form 18 July 2016
Accepted 21 July 2016
Available online 22 July 2016

Keywords:
Surfactants
Glossoscolex paulistus hemoglobin
Unfolding process

ABSTRACT

Glossoscolex paulistus hemoglobin (HbGp) is an oligomeric multisubunit protein with molecular mass of 3600 kDa. In the current study, the interaction of sodium dodecyl sulfate (SDS) and cetyl trimethylammonium chloride (CTAC) surfactants with the monomer ***d*** and the whole oxy-HbGp, at pH 7.0, was investigated. For pure monomer ***d*** solution, SDS promotes the dimerization of subunit ***d***, and the monomeric and dimeric forms have sedimentation coefficient values, $s_{20,w}$, around 2.1–2.4 S and 2.9–3.2 S, respectively. Analytical ultracentrifugation (AUC) and isothermal titration calorimetry (ITC) data suggest that up to 26 DS^- anions are bound to the monomer. In the presence of CTAC, only the monomeric form is observed in solution for subunit ***d***. For the oxy-HbGp, SDS induces the dissociation into smaller subunits, such as, monomer ***d***, trimer ***abc***, and tetramer ***abcd***, and unfolding without promoting the protein aggregation. On the other hand, lower CTAC concentration promotes protein aggregation, mainly of trimer, while higher concentration induces the unfolding of dissociated species. Our study provides strong evidence that surfactant effects upon the HbGp-subunits are different, and depend on the surfactant: protein concentration ratio and the charges of surfactant headgroups.

1. Introduction

The extracellular hemoglobins known as erythrocruorins are highly cooperative respiratory macromolecules found in mollusks and annelids [1–4]. *Glossoscolex paulistus* hemoglobin (HbGp) is a respiratory protein present in the earthworm found in the regions of Piracicaba and Rio Claro cities in the state of São Paulo, Brazil [5,6]. HbGp has a molecular mass of 3600 kDa, and its oligomeric structure is composed of heme-containing globin-like chains (144 subunits) and 36 additional polypeptide chains lacking a heme group, and named linkers [7,8]. These classes of proteins present special properties, such as, a high resistance to autoxidation [9], high oligomeric stability when exposed to denaturant agents [10–12], and high Hill coefficient [13,14], as compared to mammalian hemoglobins. These properties make these proteins an interesting and important system of study [15–17], being also an advantage in biomedical applications [16–19]. Other extracellular proteins, such as the *Lumbricus terrestris* (HbLt) [17] and the *Arenicola marina* (HbAm) hemoglobins [16,19], have been studied regarding their potential use as blood substitutes.

The structure of these hemoglobins was proposed by Vinogradov [20], which assumes that the whole protein is composed by twelve protomers, each constituted by a dodecamer of globin chains and a trimer of linkers, $[(\boldsymbol{abcd})_3\boldsymbol{L}_3]$ [8,20,21]. Here ***a***–***d*** are globin chains forming an asymmetric tetramer ***abcd***, composed of a disulfide bonded trimer ***abc*** and a monomeric subunit ***d***. Three linkers chains, $\boldsymbol{L}_1$–$\boldsymbol{L}_3$ complete the native protomer structure. Recently, HbGp crystal structure studies [8,22] have also suggested a strong similarity between HbGp and HbLt, both belonging to type 1, where the two hexagonal layers forming the bilayer are rotated by 16°, one relative to another. The Scheme 1 displays the HbGp oligomeric structure and its subunits.

The surfactants are amphiphilic molecules with application in several processes, such as, extraction of proteins from cell membranes, protein solubilization, transport of metabolites in body fluids and protein unfolding and aggregation processes [23–25]. Surfactant-protein interaction studies are relevant for the understanding of the mechanisms of interactions of these amphiphilic molecules with biomolecules and the forces that maintain the protein folded structure [23,26]. Previous studies have shown that the unfolding process induced by surfactants is characterized by different phenomena, due to the fact that proteins interact differ-

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http://dx.doi.org/10.1016/j.ijbiomac.2016.07.072
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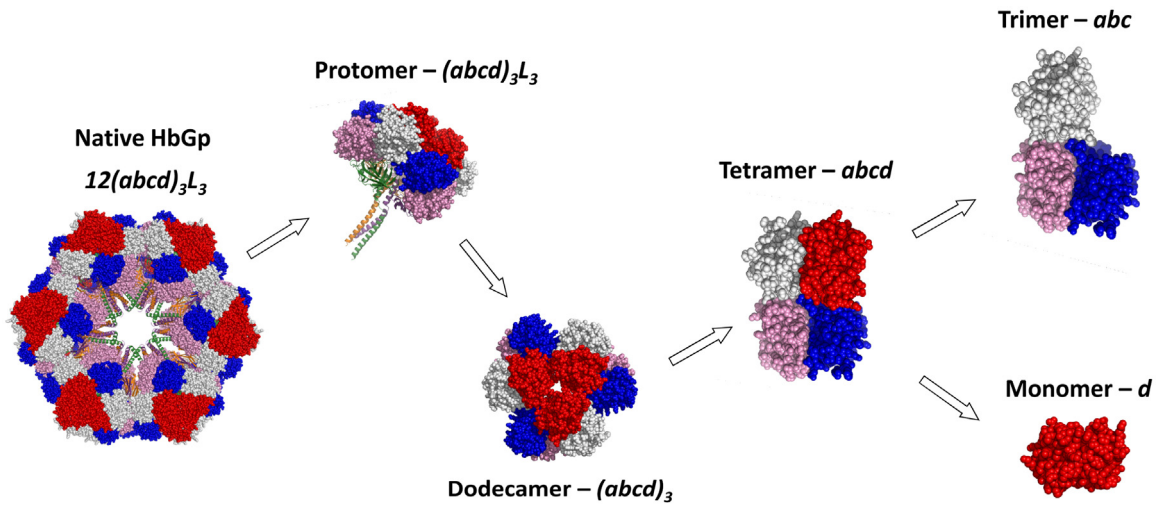

Scheme 1. Structural hierarchy of giant extracellular hemoglobin of *Glossoscolex paulistus*, HbGp. The figure was adapted from the reference [22]. PDB code: 4U8U.

ently with the monomeric and the micellar forms of surfactants [24]. Recent studies displayed that the addition of surfactant (CTAC and SDS) promotes a significant loss in the HbGp thermal stability and different phenomena, such as, aggregation, dissociation and unfolding processes [27–30]. Dynamic light scattering (DLS) data show that low CTAC concentration, at pH 7.0, promotes a shift in the unfolding critical temperature from 52 to 41 °C and a significant increase of the size of the scattering particle in solution (aggregates formation, [30]). Spectroscopic studies report that the further increase of cationic surfactant concentration induces the solubilization of large HbGp aggregates, followed by protein oligomeric dissociation and denaturation [28]. On the other hand, the addition of SDS does not promote the HbGp aggregation process, at pH 7.0, and high surfactant concentration induces the oligomeric dissociation and unfolding processes [29]. MALDI-TOF-MS studies show that CTAC interacts strongly with the HbGp, and up to 10 CTA^+ cations, at pH 7.0, are bound in the protein surface [31].

Although, several studies have been reported in the literature upon the HbGp-surfactant interaction, the characterization of HbGp subunits present in the solution, as a function of SDS and CTAC concentrations, has not been performed yet. Moreover, the information regarding the aggregation process is very limited and incomplete. For example, it is not clear if the aggregation process occurs from the whole protein or from dissociated HbGp subunits. Which HbGp subunits are more resistant to aggregation? Our present studies aim to characterize the aggregation process, the composition of solution, the differences of the HbGp unfolding process induced by CTAC and SDS. The quantification of the subunits contributions in the solution will show which HbGp species aggregate in the presence of the surfactants. Thus, this work provides more details on the HbGp surfactant-protein interaction and the subunits stabilities in the presence of ionic surfactants.

In this paper, analytical ultracentrifugation (AUC), isothermal titration calorimetry (ITC) and DLS were used to characterize the composition of the monomer ***d*** solution, as a function of SDS and CTAC concentration, at 20–25 °C, pH 7.0. AUC studies were also developed for the whole HbGp under similar surfactant concentration conditions. These studies aim to contribute to the understanding of the oligomeric dissociation, denaturation and aggregation processes. Due to the complexity of the HbGp dissociation process, in the presence of surfactant, the effect of CTAC and SDS upon the HbGp isolated subunit ***d*** was also investigated. In this way, our results, presented in Sections 3.1 and 3.2, allowed to characterize the different species in the solution, respectively, for the monomer ***d*** and the whole HbGp, as a function of surfactant concentration. The present study displays an advance in the understanding of the effects of surfactants in the oligomeric stability of a complex protein.

2. Materials and methods

2.1. Protein extraction and purification

HbGp was prepared using freshly drawn blood from the worms. HbGp solution was centrifuged at 5000 rpm for 10 min, at 4 °C. The sample was filtered (Mw cut-off 30 kDa) and centrifuged at 52,000 rpm, at 4 °C, for 3 h. The pellet was resuspended in a minimum amount of 0.1 mol/L Tris-HCl buffer, at pH 7.0. Chromatography at pH 7.0 in a Sephadex G-200 column gave the samples used in the experiments [28,32]. All concentrations were determined spectrophotometrically in a UV-1601 PC spectrophotometer (Shimadzu, Japan), using the molar absorption for oxy-HbGp of $\varepsilon_{415nm} = 5.5 \pm 0.8$ $(mg/mL)^{-1}cm^{-1}$ [11,28]. The final protein concentration in our stock solution was in the range 15–40 mg/mL, in Tris-HCl 0.1 mol/L buffer, pH 7.0.

2.2. AUC experiments

AUC experiments were performed in a Beckman Optima XL-A analytical ultracentrifuge. Sedimentation velocity (SV) experiments were carried out at monomer ***d*** concentrations from 70 up to 210 μg/mL and for oxy-HbGp in the concentration range from 100 to 300 μg/mL. The samples were dialyzed against tris–HCl 100 mmol/L buffer, pH 7.0, containing 50 mmol/L NaCl, and the dialysate was used as reference solution in all experiments. The samples were exposed to the different SDS and CTAC concentrations, in the ranges from 0.0 to 1.0 mmol/L, and from 0.0 to 4.0 mmol/L, respectively. The two surfactants were added to the protein solutions two hours before the experiments.

The SV experiments were performed at 20 °C, using a rotor speed between 15,000 and 40,000 rpm (An60Ti rotor), and the scan data acquisition was measured at 415 nm, in 7 min intervals for each curve. Absorbance data were collected using a radial step size of 0.003 cm. The rotor temperature was equilibrated to the running temperature prior to spinning of the rotor.