ELSEVIER

Biophysical Chemistry 114 (2005) 253–260

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Autoxidation studies of extracellular hemoglobin of *Glossoscolex paulistus* at pH 9: cyanide and hydroxyl effect

Alessandra Lima Poli, Leonardo Marmo Moreira, Ángel Alberto Hidalgo, Hidetake Imasato*

Instituto de Química de São Carlos, Universidade de São Paulo, P.O. Box 780, 13560-970, São Carlos, SP, Brazil

Received 29 July 2004; received in revised form 15 December 2004; accepted 17 December 2004
Available online 15 January 2005

Abstract

The complex oligomeric assembly of the hemoglobin subunits may influence the autoxidation rate. To understand this relation, the rate of autoxidation was studied at pH 9.0, where the *Glossoscolex paulistus* Hemoglobin (GpHb) dissociates. At alkaline pH, this hemoglobin is dissociated into monomers, trimers and tetramers, allowing the study of the integral protein and monomer subunit autoxidation on independent experiments. The autoxidation rate was evaluated in the presence and absence of cyanide (CN^-), a strong field ligand to the ferric ion. The oxidation kinetic was monitored using the UV–vis absorption at 415 nm, and resulted in: i) bi-exponential kinetics for the whole hemoglobin (indicating a fast and a slow oxidative process) and ii) mono-exponential for the monomer (indicating a single process). To understand the specific characteristics of each autoxidation process, Arrhenius plots allowed the determination of the activation energy. The experimental results indicate for the whole hemoglobin in the absence of CN^- an activation energy of 150 ± 10 kJ mol^{-1} for the fast and the slow processes. Under the same conditions the monomer displayed an activation energy of 160 ± 10 kJ mol^{-1}, very close to the value obtained for the integral protein. The pseudo-second order rate constant for the whole protein autoxidation by CN^- showed two different behaviors characterized by a rate constant $k'_{CN1}=0.11\pm0.02$ s^{-1} mol^{-1} L for CN^- concentrations lower than 0.012 mol L^{-1}; and $k''_{CN1}=0.76\pm0.04$ s^{-1} mol^{-1} L at higher concentrations for the fast process, while the slow process remain constant with $k_{CN2}=0.033\pm0.002$ s^{-1} mol^{-1} L. The monomer has a characteristic rate constant of 0.041 ± 0.002 s^{-1} mol^{-1} L for all cyanide concentrations. Comparing the results for the slow process of the whole hemoglobin and the oxidation of the monomer, it is possible to infer that the slow process has a strong contribution of the monomer in the whole hemoglobin kinetic. Moreover, as disulfide linkers sustain the trimer assembly, cooperativity may explain the higher kinetic constant for this subunit.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Extracellular hemoglobin; Hemoglobin autoxidation; Oligomeric assembly

1. Introduction

The extracellular hemoglobin of *Glossoscolex paulistus* (GpHb) has similar properties of other annelid hemoglobins, such as molecular weight near to 3.1×10^6 Da [1] and a hexagonal double-layered (HBL) oligomeric structure [2]. The hemoglobin of *Lumbricus terrestris* is one of the most studied in this class of proteins [3–7], which is constituted by four major globin chains *a*, *b*, *c* and *d*. These chains occur in equimolar amounts, chains *a*, *b* and *c* forming a disulfide-linked trimer, while chain *d* is an isolated monomer. Furthermore, measurements of the ratio protein/heme mass indicate the presence of some amount of polypeptides named linkers that lack the heme group [8] and are essential in preserving the integrity of the hemoglobin molecule [9].

Several observations, suggestions and predictions made with regard to the quaternary structure of the *abcd* tetrameric unit of *L. terrestris* has been subsequently borne out by the 5.5 Å resolution crystal structure determination of the whole particle [10]. The analysis of this crystallographic data shows that the whole molecule consists of 144 globin

Abbreviations: GpHb, *Glossoscolex paulistus* Hemoglobin; met-HbGp, methemoglobin of *Glossoscolex paulistus*; oxy-HbGp, oxyhemoglobin of *Glossoscolex paulistus*.

* Corresponding author. Tel.: +55 16 3373 9955; fax: +55 16 3373 9982.

E-mail address: hidetake@iqsc.usp.br (H. Imasato).

0301-4622/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2004.12.041

and 36 linker chains. The globin subunits in the particle were identified by their general globular geometry and are arranged into 12 substructures each of which is composed of three (*abcd*) tetrameric units. The *linker* chain assembly was roughly described as a globular folded region located between two hexagonal layers with three emerging rodlike portions oriented towards the center of the HBL, associated to the central cryomicroscopic dense regions [11].

Zhu et al. [12] studied the assembly of the hemoglobin of *L. terrestris* at neutral pH and found that the assembly (association and dissociation of the subunits) depends on the oxidation state. Particularly, oxidation of the $(abcd)_4$ complex with ferricyanide causes complete dissociation into *d* monomer and *abc* trimer, but addition of CN^- maintains the $(abcd)_4$ complex or reassembles the oxidized subunits. The assembly properties of the oxyhemoglobin of *G. paulistus* at alkaline pH were studied by Imasato and Agustinho [13,14]. The experimental results shows that the oxyhemoglobin dissociates at pH 9.0 into dimer of tetramers-$(abcd)_2$, trimers-*abc*, and monomers *d* [13] while the met form dissociates just into trimers and monomers [14]. Recently, Gelamo et al. [15] showed clearly by SAXS (small angle X-ray scattering), that radius of gyration of the oxy-HbGp and the met-HbGp decreases from 110 Å at pH 7.0 to 31 Å at pH 9.0, indicating the dissociation of the complex even at high concentration, 3 $mg \cdot ml^{-1}$ ($\sim 1 \times 10^{-4}$ mol L^{-1}). The assembly of the subunits has an important function in the O_2 binding for the Human hemoglobin [16]. In fact, studies on the alosteric effect of the Human hemoglobin indicate that cooperativity is mediated through the interaction of the subunits.

Many efforts have been devoted to obtain recombinant heme proteins that present good stability against autoxidation, maintaining low oxygen affinity and higher cooperativity [17–20]. These features are important for efficient oxygen carriers. The oxygenated form of hemoglobin, as well as myoglobin, is known to be oxidized easily to ferric (III) met form, which cannot bind molecular oxygen and is therefore physiologically inactive [21,22]. The hemoglobin autoxidation is related to the unequal distribution of electrons along the $Fe-O_2$ bond. This confers a superoxide character to the bound oxygen, allowing the ligand to dissociate as superoxide anion. The presence of CN^- and OH^- increases the autoxidation because both are weak ligands to the ferrous ion and strong ligands to the ferric ion. The action of both ligands, CN^- and OH^-, is to promote the oxidation by stabilizing the ferric state [23–26]. This low affinity of cyanide for ferrous porphyrins is caused by the repulsion between the high electron density on the iron and the negatively charged cyanide [26].

Two mechanisms of autoxidation of hemoglobins and myoglobins have been proposed [27]: internal and external sphere. Gonzalez et al. [28] have discussed several reaction pathways that may affect the rate constant of the net reaction. In this way, the rate constant may be affected by the experimental conditions, pH, oxygen pressure, presence of ferric ligands, etc. Among the factors influencing the rate of autoxidation of MbO_2 and HbO_2, the effect of pH has been widely investigated [21,22,27].

In this work, we show experimental results on the autoxidation of *G. paulistus* whole hemoglobin comparing with the isolated *d* monomer autoxidation in alkaline medium. To induce the autoxidation, the hydroxyl and cyanide ions were used as ligands to the metallic center. The main purpose of this work is to contribute on the understanding of the autoxidation rate of the extracellular hemoglobins of the giant worm *G. paulistus* and the relation with the oligomeric assembly.

2. Methods and materials

2.1. The hemoglobin and monomer preparations

The hemoglobin of *G. paulistus* was prepared using freshly drawn blood from worms. The blood sample was purified by ultra centrifugation and gel filtration in Sephadex G-200 column at pH 7.0 [13]. The oxymonomer was obtained from previously purified hemoglobin using the gel filtration in Sephadex G-200 at pH 9 [13].

2.2. Autoxidation rate measurements

The autoxidation rate of dissociated oligomeric hemoglobin and monomeric fraction was measured as a function of temperature in a range between 36 °C and 43.5 °C, pH 9.0 using Tris–HCl buffer. The experiments in the presence of different amount of cyanide were performed at 38 °C, at pH 9. In each measurement, a given volume of buffer was equilibrated in water bath at the desired temperature previously to the dilution of oxyhemoglobin to the working concentration (ca. 2.5–3.0 μmol L^{-1} based on $\varepsilon_{415\ nm}$=129 $mmol^{-1}\ L^{-1}\ cm^{-1}$ [13]). The reaction started immediately after dilution of hemoglobin. For the studies of the cyanide effects, the experiments were started after adding an aliquot of cyanide concentrated solution.

The resonant scattering was used to control the possible reassembly of the subunits during the study of the integral protein autoxidation in the presence of cyanide. Light scattering at 310 nm was measured at 90° in a spectrofluorometer by selecting the same wavelength for both excitation and emission monocromators. Measurements were corrected for the light scattered by the buffer. The light scattering for a sample of $(2.6 \pm 0.1) \times 10^{-6}$ mol L^{-1} concentration was approximately 10 times higher than the scattering of the buffer.

2.3. Determination of rate constants

In order to obtain the rate constants of autoxidation, the absorbance at 415 nm (A_{415}) was monitored in time. The collected data were fitted to a combination of first order

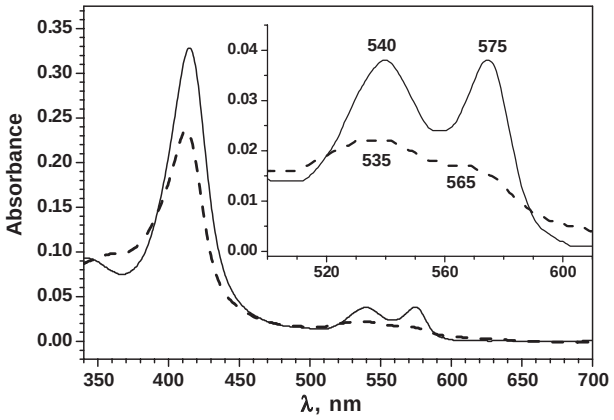

Fig. 1. Optical absorption spectra of oxyhemoglobin of *Glossoscolex paulistus* (solid line) in 20 mmol L^{-1} Tris–HCl buffer (pH 9), 42 °C, 2.5 μmol L^{-1} heme based and spectral changes into hemichrome (dashed line) due to the autoxidation.

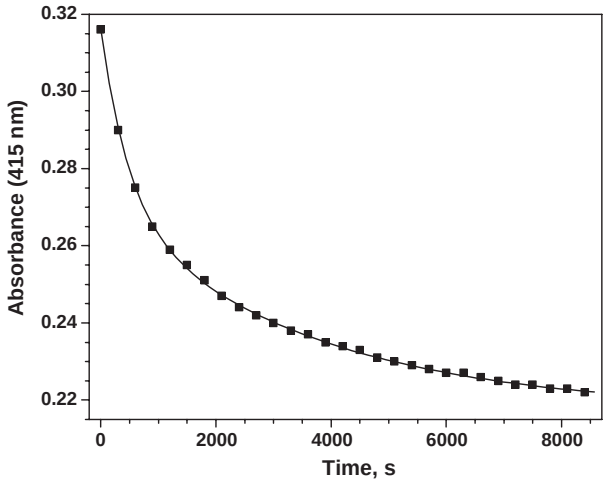

Fig. 2. Autoxidation of *Glossoscolex paulistus* oxyhemoglobin in Tris–HCl 20 mmol L^{-1} buffer at pH 9, 43.5 °C and 2.5 μmol L^{-1} heme based.

kinetics [21] with two rate constants; characterizing a fast process and a slow one.

$$A_t = \Delta A_{\max_1} \exp(-k_{\mathrm{obs}_1} t) + \Delta A_{\max_2} \exp(-k_{\mathrm{obs}_2} t) + A_\infty \quad (1)$$

In this equation, k_{obs_1} and k_{obs_2} represent first order rate constants to the fast and slow autoxidation processes, respectively. $\Delta A_{\max_1}$ and $\Delta A_{\max_2}$ are the variation of absorbance ($A_{t=0} - A_\infty$), which corresponds to the total oxidation of the rapid and slow reacting hemes, respectively. A_t is the absorbance at time t and A_∞ is absorbance at infinite time. The oxidation of the integral hemoglobin needed the combination of two exponentials, while the isolated monomer reaction fitted well with a mono-exponential.

To avoid too many free parameters during the fitting session, A_∞ values were fixed, employing the estimated values based on long time experiments, where it was determined the ratio $A_\infty / A_{t=0}$. To establish the experimental error, three independent experiments were carried out and the mean value of the fitted constants was used.

3. Results

3.1. Autoxidation at pH 9.0

The spectral changes that characterize *G. paulistus* oxidation at pH 9.0 can be observed in Fig. 1: i) the shift of the Soret band from 415 nm to 412 nm; ii) the shift of the β and α bands from 540 nm and 575 nm to 535 nm and 565 nm, respectively. The final spectrum corresponds to the hemichrome, where the sixth ligand of the iron becomes the imidazole of distal histidine residue [29–31]. Thus, during the process, the iron is converted to the ferric met form, and the hydroxyl ion remains bound to the Fe(III) at the sixth-coordinate position to form hydroxide-methemoglobin [32,33].

In fact, Tsuruga et al. [22] presented results that imply a nucleophilic displacement of O_2^- from the β chain of human oxyhemoglobin by entering a water molecule or hydroxyl ion. This process is the rate-limiting step; and the subsequent conversion of the met form into hemichrome is very fast, i.e., $k_{His} \gg k_0$ (see Scheme 1). A similar process may be associated to the autoxidation of *G. paulistus* hemoglobin.

Scheme 1 shows the global reaction at pH 9.0; nevertheless, to understand the detailed aqueous autoxidation mechanism, experiments at different pH are necessary

$$\mathrm{Fe(II)(O_2) + OH^- \xrightarrow{k_0} Fe(III)(OH^-) + O_2^- \xrightarrow{k_{His}} Fe(III)(His)}$$

Scheme 1.

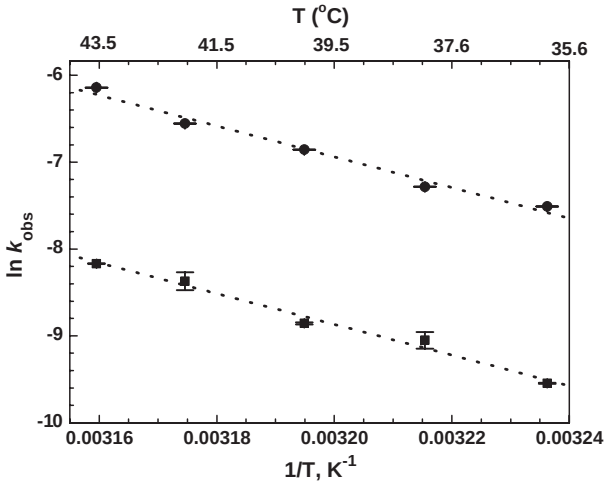

Fig. 3. Arrhenius plot for autoxidation of whole *Glossoscolex paulistus* hemoglobin: first component k_{obs_1} (●) and second component k_{obs_2} (■). The autoxidation rates of oxyhemoglobin were measured at 20 mmol L^{-1} Tris–HCl buffer (pH 9). Error bars were determined from three independent experiments.

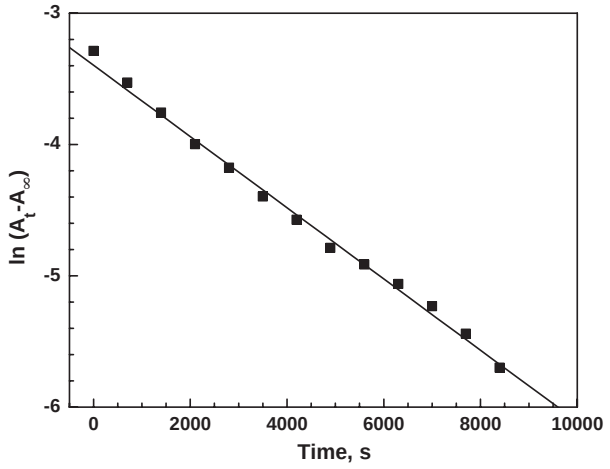

Fig. 4. Plot of first-order kinetic for autoxidation of *Glossoscolex paulistus* monomer, chain *d*, in Tris–HCl 20 mmol L^{-1} buffer at pH 9, 43.5 °C.

according to the results on other hemoproteins [34–37]. Experiments of other authors indicate that increasing the hydrogen ionic concentration (acid pH), the reaction is assisted by the H^+ ion (proton-relay mechanism [22]). Since of GpHb monomer presents the distal histidine, as in the human Hb case, a similar process may take place. At alkaline pH, the nucleophilic displacement by OH^- is observed in the human Hb. Thus, both ions H^+ and OH^- may enhance the autoxidation rate in the GpHb. However, the experimental work presented in this article at just one pH is rather limiting [27] and more experiments at different pH are being carried out to elucidate the mechanism of aqueous autoxidation and the oligomeric assembly of the GpHb.

The autoxidation of the whole *G. paulistus* hemoglobin monitored spectrophotometrically at 415 nm (Fig. 2) showed a complex behavior as observed earlier with the human Hb at pH 6.5 [21]. The oxidation of the GpHb was fitted by combining two first order kinetics, Fig. 2 displays one example at 43.5 °C. Any attempt to fit the experimental

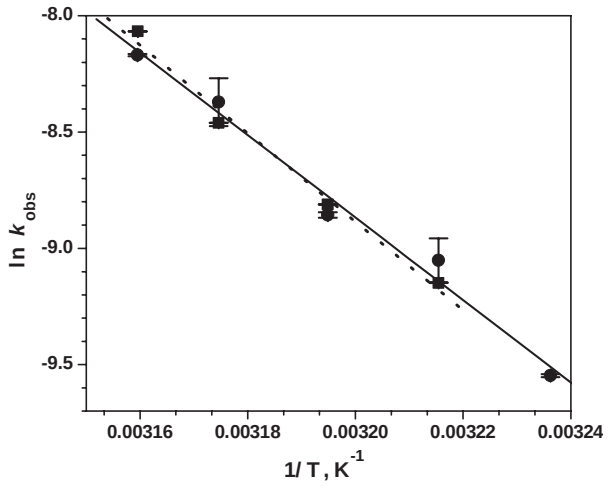

Fig. 5. Arrhenius plot of rate constants of monomer (■) and the second component k_{obs_2} (●) of whole *Glossoscolex paulistus* hemoglobin. Error bars were determined from three independent experiments.

Table 1
Autoxidation rate constants in alkaline pH of hemoglobins and myoglobins

	Globin		k_{obs} (h^{-1})
Glossoscolex paulistus	Integral	Fast process	2.4
		Slow process	0.36
	Monomer *d*		0.36
Human hemoglobin	Chains α		0.052 [17]
	Chains β		0.089 [17]
Bovine oxymyoglobin			0.011 [32]
Perinereis brevicirris	Subunit 1		1.32 [33]
	Subunit 2		0.17 [33]

data with just one kinetic process were completely unsuccessful. Fluorescence and chromatographic studies indicated that the GpHb is dissociated at alkaline pH [13]. Then, the two kinetic processes may be related to dissociated subunits. In fact, the observed nonequivalent autoxidation is related to two populations of heme groups each one reacting according to the observed k_{obs_1} and k_{obs_2}, respectively.

The rate constants values, k_{obs_1} and k_{obs_2} obtained at different temperatures, are presented as Arrhenius plots in Fig. 3. The activation energies for the autoxidation reactions were evaluated to be $E_1=E_2=150\pm10$ kJ mol^{-1} related; to the first and second components, respectively.

Fig. 4 shows an example of the autoxidation data obtained for the isolated *d* fraction of *G. paulistus* hemoglobin at 43.5 °C. The logarithmic plot ($\ln(A_t-A_\infty)$ vs. time), clearly shows a linear relation, indicating the presence of just one kinetic process. From the slope, the observed first order rate constants were determined at different temperatures; and the corresponding Arrhenius plot is shown in Fig. 5. In this case, the activation energy for the isolated monomer was 160 ± 10 kJ mol^{-1}.

The components of *G. paulistus* hemoglobin showed rate constants (Table 1) higher than the human hemoglobin isolated chains [22] and bovine oxymyoglobin [38], in

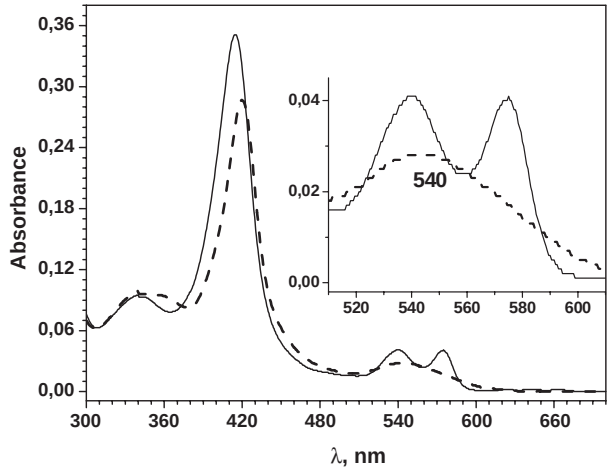

Fig. 6. Spectral change upon the autoxidation of *Glossoscolex paulistus* hemoglobin (2.6 μmol L^{-1} heme based) in the presence of 0.023 mol L^{-1} cyanide, Tris–HCl buffer 20 mmol L^{-1} (pH 9.0) at 38 °C. Oxyhemoglobin (solid line) and methemoglobin-cyanide (dashed line).

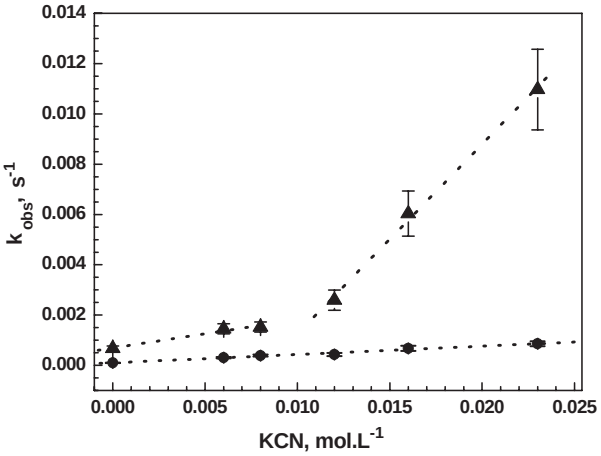

Fig. 7. The observed first order rate constants, k_{obs_1} (▲) and k_{obs_2} (●), as a function of potassium cyanide concentrations. The slope of the graph corresponds to k'_{CN1}, k''_{CN1} and k_{CN2}, the rate constants for the cyanide-induced oxidation of hemoglobin in s^{-1} mol^{-1} L. Conditions: $(2.6\pm0.1)\times10^{-6}$ mol L^{-1} hemoglobin in 20 mmol L^{-1} Tris–HCl buffer, pH 9.0 at 38 °C. Error bars were determined from three independent experiments.

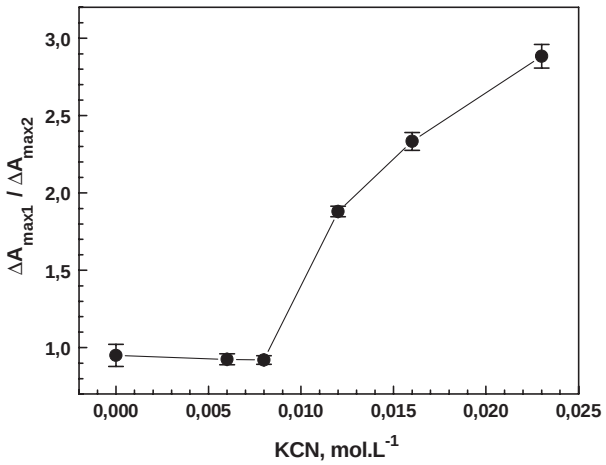

Fig. 8. Plot of pre-exponentials factors ratio ΔA_{max_1} (fast process) and ΔA_{max_2} (slow process) as a function of cyanide concentration. Error bars were determined from three independent experiments.

agreement with Suzuki et al. [34]. Suzuki et al. observed two subunits isolated of *Perinereis brevicirris* extracellular hemoglobin with much higher autoxidation rates than that of bovine oxymyoglobin at alkaline pH. It seems that the components of *G. paulistus* hemoglobin present the hydrophobic pocket more accessible to the solvent than that of mammalian hemoproteins.

3.2. Autoxidation in the presence of cyanide

Fig. 6 shows the spectral changes due to the autoxidation of GpHb in the presence of potassium cyanide. In the presence of cyanide, the process is characterized by: i) the shift of the Soret band from 415 nm to 420 nm and; ii) the change of the α and β bands from 575 nm and 540 nm to only one broad band at 540 nm. The last spectrum corresponds to a cyanide–metHb complex (CN–metHb) [14].

As in the absence of cyanide, the kinetic behavior was complex and a combination of two first-order processes were necessary to achieve the best fitting for the whole GpHb. The rate constants determined at different KCN concentrations allowed the determination of the pseudo-second order constant k_{CN} as indicated in Fig. 7. Clearly, the fast process k_{obs_1} shows two different constants. For concentrations lower than 0.010 mol L^{-1} was $k'_{CN1}=0.11\pm0.02$ s^{-1} mol^{-1} L and for concentrations higher than 0.12 mol L^{-1} was $k''_{CN1}=0.76\pm0.04$ s^{-1} mol^{-1} L. k_{CN2} remain unchanged for all concentrations, equal to 0.033 ± 0.002 s^{-1} mol^{-1} L (Table 2). These observations can be correlated with the analysis of the ratio of the pre-exponentials factors $\Delta A_{max_1}/\Delta A_{max_2}$ (see Eq. (1)). This ratio is constant and close to one until 0,008 mol L^{-1} of CN^-; increasing to 3 at 0.023 mol L^{-1} of CN^- (Fig. 8).

Zhu et al. observed that the presence of cyanide cause re-assembly of the subunits of the *L. terrestris* hemoglobin [12]; in this way the autoxidation kinetic of HbGp in the presence of cyanide was studied also by light scattering at 90°. Silva and coworkers [39] used this experiment to monitor the assembly of the subunits of the HbGp under hydrostatic pressure. Fig. 9 shows the intensity collected at 90° for both the excitation and emission monocromators at 310 nm. We selected two cyanide concentrations for testing

Table 2
Rate constants for the displacing oxidation of *Glossoscolex paulistus* hemoglobin by cyanide

Glossoscolex paulistus hemoglobin			k_{CN} ($s^{-1}mol^{-1}$ L)
Integral	Fast process	k'_{CN1}	0.11±0.02
		k''_{CN1}	0.76±0.04
	Slow process (k_{CN2})		0.033±0.002
Monomer *d*			0.041±0.002

k'_{CN1} was obtained using the data for cyanide concentration lower than 0.010 mol L^{-1} and k''_{CN1} for concentration higher than 0.010 mol L^{-1}.

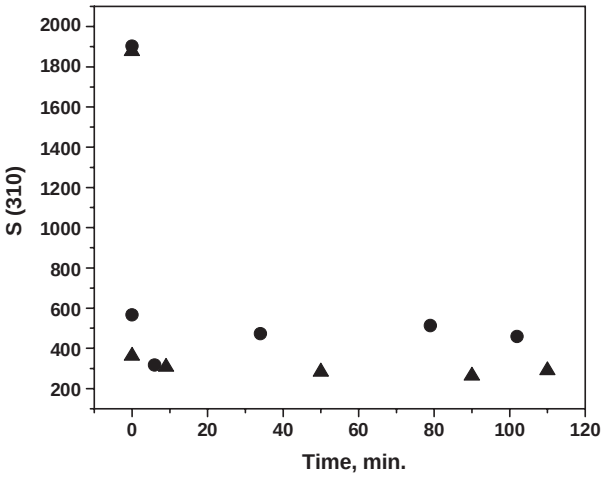

Fig. 9. Light scattering of the integral hemoglobin of *G. paulistus* measured at 90° of the incident light (=310 nm) during the autoxidation process (2.6 μmol L^{-1} heme based) in the presence of 0.006 mol L^{-1} (●) and 0.016 mol L^{-1} (▲) of cyanide, Tris–HCl buffer 20 mmol L^{-1} (pH 9.0) at 20 °C.

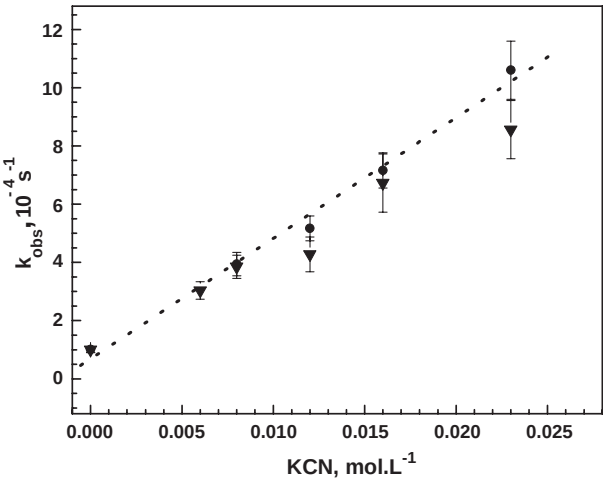

Fig. 10. The observed rate constants plot of second component, k_{obs_2} (▼) of whole *Glossoscolex paulistus* hemoglobin and of monomer (●) as a function of cyanide concentrations. Error bars were determined from three independent experiments.

the resonant scattering, both are related with the change in slope observed in the k_{obs} of the fast autoxidation process (0.006 mol L^{-1} and 0.016 mol L^{-1}). The experimental data with both concentrations have similar behaviors, see Fig. 9; after the addition of cyanide (first point) the intensity decays to a value that remains constant till the end of the experiment. The decay associated to the addition of the cyanide may be related to further dissociation promoted by the presence of the cyanide ion (probably due to a change on the ionic strength), however, the fact that after the first point the intensity remains constant, gives a strong evidence that the particle that produce the scattering remains the same; a clear indication that the hemoglobin assembly does not change during the autoxidation process.

The oxidation of the monomer in the presence of cyanide was also analyzed (Fig. 10). As in the buffer experiments, just one rate constant was enough to describe the oxidation kinetic experiment (UV–vis absorption at 415 nm vs. time). The dependence of k_{obs} for the monomer with KCN concentration is shown in Fig. 9 and the pseudo-second order rate determined from these data was 0.041 ± 0.002 mol^{-1} L s^{-1}.

4. Discussion

According to Figs. 5 and 9 it is possible to verify that the observed rate constant of the slow process of the whole protein and of the isolated monomer *d* are very well correlated either in the absence or in the presence of cyanide. These two information indicate that the monomer autoxidation dominates the slow process, and the trimer in the integral protein dominates the fast process. Assuming that the fast process could be associated to the trimer, this autoxidation can be rationalized considering two features: i) the possible cooperative effect in the trimer and ii) the lowest oxygen affinity for the trimer in relation to monomer.

Cooperativity effect is usually observed for the oxygen binding in the tetrameric hemoglobin as well as in trimer of worm hemoglobin [40]. The binding of the first cyanide to the first heme of the trimer, facilitates the bonding of cyanide to the hemes located in other chains, i.e., the binding of the first cyanide can be propagated to the other chains through conformational changes in the first oxidized chain, rendering the other subunits more susceptible to oxidation. This cooperativity can be based on the fact that chains *a, b* and *c* are linked by disulfide bonds and according to Ackers and coworkers [16], the interaction between the subunits is the key property for cooperativity. The transition from the oxy form to the cyan-met form presents a highly irreversible character, different from the equilibrium of O_2 binding usually found in cooperativity studies. This irreversibility renders difficult the quantification of the cooperativity in the reaction studied in this article manifested as a higher rate constant.

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 the oxygen affinity [41]. In fact, the increase autoxidation rates observed appear to be due to a decrease in oxygen affinity and an increase in solvent anion accessibility to the distal pocket [23]. Furthermore, Fushitani at al. described that the trimer of *L. terrestris* presents lower affinity to oxygen (log P_{50}~0.7 pH 7.0) than monomer (log P_{50}~−0.2, pH 7.0) [40]. This behavior is related with the effects of the presence of distal histidine, i.e., steric accessibility, hydrogen bonding, and local polarity that play relevant roles in regulating the rate of heme iron autoxidation [18,20,23,24,35–37,42].

Recently, it was defined the chemical structure of the monomeric chain of *G. paulistus* and the modeling was performed [43]. The chain *d* of this hemoglobin has a tryptophan residue in the B10 position and the distal residue is a histidine (E7). Among the distal heme pocket residues, the B10 is known to be very relevant for the O_2 binding, in addition to the significant influence of the E7 residue [42]. Kamimura et al. described that it is possible that the B10–Phe at position 32 is stabilizing the bound O_2 in *Tokunagayusurika akamusi* hemoglobin V [35]. Carver et al. [17], Brantley et al. [18] and Jeong [19] showed that the mutation at the B10, the exchange of the leucine by others residues, in globin molecules increased the stability against autoxidation or suppressed completely the autoxidation showing the straight correlations of the autoxidation and affinity to the oxygen molecule.

It is worth to mention that the monomer of *G. paulistus d*-chain presented 55% of sequence identity with the *L. terrestris d*-chain (d1) [43]. Since the trimer chemical structure of *G. paulistus* is not solved yet, the *L. terrestris* trimer sequence could be used to give information about the system, helping to explain the lower stability of the trimer in the autoxidation process in relation to the monomer. In fact, chain *c* of the trimer is the unique that presents the leucine

residue in B10 position similarly to the human hemoglobin. In this way, the leucine could be the pivot of the low stability of trimer due to the non-polar properties of its side chain. On the other hand, the *d* monomer of *G. paulistus* presents a residue of tryptophan in the B10 position, which has high polarity and a side group with great volume. Therefore, the tryptophan could stabilize O_2 as a sixth ligand of the monomer heme in a similar way of phenylalanine [35].

Since the trimer structure is held by covalent linkage, and chain *c* presents the leucine in B10 position, it could be the first to oxidize. The effect of this change would propagate to the other chains making them more susceptible to the oxidation by cooperative effect. Other studies to test this hypothesis are being developed in our group.

Finally, the evaluated activation energies for the autoxidation reactions were $E_1=E_2=150\pm10$ kJ mol^{-1} for the first and second components of whole hemoglobin and isolated monomer, respectively, and 160 ± 10 kJ mol^{-1} from the isolated monomer *d* data. These values are similar, suggesting that the same autoxidation mechanism takes place for all hemoglobin components.

5. Conclusions

The autoxidation of *G. paulistus* whole hemoglobin in the presence of the anionic ligands cyanide and hydroxyl showed a complex behavior described by a combination of two first-order kinetics. The slower process was identified to be the monomer autoxidation and the fast process may be attributed to the autoxidation of the trimer, i.e., the monomer is more stable against the autoxidation. The cyanide presented a larger effect in the autoxidation rate than the hydroxyl probably because cyanide is a stronger ligand than hydroxyl to ferric ion. However, in both experiments, in the presence of the hydroxyl and cyanide ions, it was possible to discriminate two populations of globin in the whole hemoglobin.

Acknowledgements

The authors are indebted to Prof. M. Tabak for the fruitful discussions and to the Brazilian funding agencies FAPESP, CNPq and CAPES for the student grants to A.L. Poli, L.M. Moreira and Technological Development grant to A.A. Hidalgo.

References

[1] M.C.P. Costa, C.F.S. Bonafé, N.C. Meirelles, F. Galembeck, Sedimentation coefficient and minimum molecular-weight of extracellular hemoglobin of *Glossoscolex paulistus Oligochaeta*, Br. J. Med. Res. 21 (1988) 115–118.

[2] N.C. Meirelles, B. Oliveira, A.R. Oliveira, E. de Paula, S. Marangoni, G.M. Rennebeck, Erythrocruorin of *Glossoscolex-paulistus* (*Oligochaeta, Glossocolecidae)*—dissociation at alkaline pH and its ligand properties as revealed by chemical, immunochemical and electron-microscopy studies, Comp. Biochem. Physiol. 88A (1987) 337–379.

[3] P.D. Martin, A.R. Kuchumov, B.R. Green, R.W.A. Oliver, E.H. Braswell, J.S. Wall, S.N. Vinogradov, Mass spectrometric composition and molecular mass of *Lumbricus terrestris* hemoglobin: a refined model of its quaternary structure, J. Mol. Biol. 255 (1996) 154–169.

[4] A.R. Kuchumov, J.C. Taveau, J.N. Lamy, J.S. Wall, R.E. Weber, S.N. Vinogradov, The roles of linkers in the reassembly of the 3.6 MDa hexagonal bilayer hemoglobin from *Lumbricus terrestris*, J. Mol. Biol. 289 (1998) 1361–1374.

[5] E. Viana, C.H.T.P. Silva, M. Tabak, H. Imasato, R.C. Garratt, A molecular model for the d chain of the giant haemoglobin from *Lumbricus terrestris* and its implications for subunit assembly, Biochim. Biophys. Acta 1383 (1998) 130–142.

[6] F. Haas, A. Kuchumov, J.C. Taveau, N. Boisset, S.N. Vinogradov, J.N. Lamy, Three-dimensional reconstruction of native and reassembled *Lumbricus terrestris* extracellular hemoglobin. Localization of the monomeric globin chains, Biochemistry 36 (1997) 7330–7338.

[7] F. Mouche, N. Boisset, P.A. Penczek, *Lumbricus terrestris* hemoglobin—the architecture of linker chains and structural variation of the central toroid, J. Struct. Biol. 133 (2001) 176–192.

[8] K. Fushitani, A.F. Riggs, Non-heme protein in the giant extracellular hemoglobin of the earthworm *Lumbricus terrestris*, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 9463–9464.

[9] S.N. Vinogradov, S.D. Lugo, M.G. Mainwaring, O.H. Kapp, A.V. Crewe, Bracelet protein: a quaternary structure proposed for the giant extracellular hemoglobin of *Lumbricus terrestris*, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 8034–8038.

[10] W.E. Royer Jr., K. Strand, M. Van Heel, W.A. Hendrickson, Structural hierarchy in erythrocruorin, the giant respiratory assemblage of annelids, Proc. Natl. Acad. Sci. 97 (2000) 7107–7111.

[11] J.C. Taveau, N. Boisset, S.N. Vinogradov, J.N. Lamy, Three-dimensional reconstruction of *Lumbricus terrestris* hemoglobin at 22 Å resolution: intramolecular localization of the globin and linker chains, J. Biol. Chem. 289 (1999) 1343–1359.

[12] H. Zhu, D.W. Ownby, C.K. Riggs, N.J. Nolasco, J.K. Stoops, A.F. Riggs, Assembly of the gigantic hemoglobin of the earthworm *Lumbricus terrestris*. Roles of subunit equilibria, non-globin linker chains, and valence of the heme iron, J. Biol. Chem. 271 (1996) 30007–30021.

[13] H. Imasato, M.H. Tinto, J.R. Perussi, M. Tabak, Fluorescence studies of extracellular hemoglobin of *Glossoscolex paulistus* obtained by gel filtration, Comp. Biochem. Physiol. 112B (1995) 217–226.

[14] S.C.M. Augustinho, M.H. Tinto, J.R. Perussi, M. Tabak, H. Imasato, Fluorescence studies of extracellular hemoglobin of *Glossoscolex paulistus* in met form obtained by sephadex gel filtration, Comp. Biochem. Physiol. 118A (1997) 171–181.

[15] E. Gelamo, R. Itri, M. Tabak, Small angle X-ray scattering (SAXS) study of the extracellular hemoglobin of *Glossoscolex paulistus*, J. Biol. Chem. 279 (2004) 33298–33305.

[16] G.K. Ackers, M.L. Doyle, D. Myers, M.A. Daugherty, Molecular code for cooperativity in hemoglobin, Science 255 (1992) 54–63.

[17] T.E. Carver, R.E. Brantley, E.W. Singleton, R.M. Arduini, M.L. Quillin, G.N. Phillips, J.S. Olson, A novel site-directed mutant of myoglobin with an unusually high O_2 affinity and low autoxidation rate, J. Biol. Chem. 267 (1992) 14443–14450.

[18] R.E. Brantley Jr., S.J. Smerdon, A.J. Wilkison, E.W. Singleton, J.S. Olson, The mechanism of autoxidation of myoglobin, J. Biol. Chem. 268 (1993) 6995–7010.

[19] S.T. Jeong, N.T. Ho, M.P. Hendrich, C. Ho, Recombinant hemoglobin(alpha 29leucine→phenylalanine, alpha 96valine→tryptophan, beta 108asparagine→lysine) exhibits low oxygen affinity and high cooperativity combined with resistance to autoxidation, Biochemistry 38 (1999) 13433–13442.

[20] T. Tada, Y. Watanabe, A. Matsuoka, M. Ikeda-Saito, K. Imai, Y. Ni-Hei, K. Shikama, African elephant myoglobin with an unusual

autoxidation behavior: comparison with the H64Q mutant of sperm whale myoglobin, Biochim. Biophys. Acta 1387 (1998) 165–176.
[21] M. Tsuruga, K. Shikama, Biphasic nature in the autoxidation reaction of human oxyhemoglobin, Biochim. Biophys. Acta 1337 (1997) 96–104.
[22] M. Tsuruga, A. Matsuoka, A. Hachimori, Y. Sugawara, K. Shikama, The molecular mechanism of autoxidation for human oxyhemoglobin. Tilting of the distal histidine causes nonequivalent oxidation in the β chain, J. Biol. Chem. 273 (1998) 8607–8615.
[23] B.A. Springer, K.D. Egeberg, S.G. Sligar, R.J. Rohlfs, A.J. Mathews, J.S. Olson, Discrimination between oxygen and carbon monoxide and inhibition of autooxidation by myoglobin, J. Biol. Chem. 264 (1989) 3057–3060.
[24] W.J. Wallace, R.A. Houtchens, J.C. Maxwell, W.S. Caughey, Mechanism of autoxidation for hemoglobin and myoglobins—promotion of superoxide production by protons and anions, J. Biol. Chem. 257 (1982) 4966–4977.
[25] M. Ikeda-Saito, H. Hori, L.A. Anderson, R.C. Prine, I.J. Pickering, G.N. George, C.R. Sanders II, R.S. Lutz, E.J. Mc Kelvey, R. Mattera, Coordination structure of the ferric heme iron in engineered distal histidine myoglobin mutants, J. Biol. Chem. 267 (1992) 22843–22852.
[26] J.A. Sigman, A.E. Pond, J.H. Dawson, Y. Lu, Engineering cytochrome *c* peroxidase into cytochrome *P*450: a proximal effect on heme-thiolate ligation, Biochemistry 38 (1999) 11122–11129.
[27] K. Shikama, The molecular mechanism of autoxidation for myoglobin and hemoglobin. A venerable puzzle, Chem. Rev. 98 (1998) 1357–1373.
[28] G. Gonzalez, M.A. Gilles-Gonzalez, E.V. Rybak-Akimova, M. Buchalova, D.H. Busch, Mechanism of autoxidation of the oxygen sensor FixL and *Aplysia* myoglobin: implications for oxygen-binding heme proteins, Biochemistry 37 (1998) 10188–10194.
[29] S.C.M. Augustinho, M.H. Tinto, H. Imasato, T.T. Tominaga, J.R. Perussi, M. Tabak, Spectroscopy studies of the met form of the extracellular hemoglobin from *Glossoscolex paulistus*, Biochim. Biophys. Acta 1298 (1996) 148–158.
[30] E.A. Rachmilewitz, J. Peisach, W.E. Blumberg, Studies on the stability of oxyhemoglobin A and its constituent chains and their derivatives, J. Biol. Chem. 246 (1971) 3356–3366.
[31] L. Vitagliano, G. Bonomi, A. Riccio, G.D. Prisco, G. Smulevich, L. Mazzarella, The oxidation process of Antartic fish hemoglobins, Eur. J. Biochem. 271 (2004) 1651–1659.
[32] Y. Satoh, K. Shikama, Autoxidation of oxymyoglobin. A nucleophilic displacement mechanism, J. Biol. Chem. 256 (1981) 10272–10275.
[33] K. Shikama, Stability properties of dioxygen–iron(II) porphyrins: an overview from simple complexes to myoglobin, Coord. Chem. Rev. 83 (1988) 73–91.
[34] T. Suzuki, M. Koboyashi, T. Furukohri, T. Gotoh, Autoxidation of extracellular oxyhaemoglobin from the *Polychaete Perinereis Brevicirris* (Grube), Comp. Biochem. Physiol. 75B (1983) 17–21.
[35] S. Kamimura, A. Matsuoka, K. Imai, K. Shikama, The swinging movement of the distal histidine residue and autoxidation reaction for midge larval hemoglobins, Eur. J. Biochem. 270 (2003) 1424–1433.
[36] K. Shikama, A. Matsuoka, *Aplysia* oxymyoglobin with an unusual stability property: kinetic analysis of the pH dependence, Biochemistry 25 (1986) 3898–3903.
[37] T. Suzuki, Y. Watanabe, M. Nagasawa, A. Matsuoka, K. Shikama, Dual nature of the distal histidine residue in the autoxidation reaction of myoglobin and hemoglobin-comparison of the H64 mutants, Eur. J. Biochem. 267 (2000) 6166–6174.
[38] Y. Sugawara, K. Shikama, Autoxidation of native oxymyoglobin. Thermodynamic analysis of the pH profile, Eur. J. Biochem. 110 (1980) 241–246.
[39] J.L. Silva, M. Villas-Boast, C.F.S. Bonafe, N.C. Meirelles, Anomalous pressure dissociation of large protein aggregates, J. Biol. Chem. 264 (1989) 15863–15868.
[40] K. Fushitani, A.F. Riggs, The extracellular hemoglobin of the earthworm, *Lumbricus terrestris*, oxygenation properties of isolated chains, trimer and reassociated product, J. Biol. Chem. 266 (1991) 10275–10281.
[41] E.E. Di Iorio, K.H. Winterhalter, A. Mansouri, W.E. Blumberg, J. Peisach, Studies on the autoxidation of hemoglobin Zurich (beta-63 E7 Arg), Eur. J. Biochem. 145 (1984) 549–554.
[42] X. Zhao, K. Vyas, B.D. Nguyen, K. Rajarathnam, G.N. La Mar, T. Li, G.N. Phillips Jr., R.F. Eich, J.S. Olson, J. Ling, D.F. Bocian, A double mutant of sperm whale myoglobin mimics the structure and function of elephant myoglobin, J. Biol. Chem. 270 (1995) 20763–20774.
[43] C.B. Cabral, H. Imsato, J.C. Rosa, H.J. Laure, C.H.T.P. da Silva, M. Tabak, R.C. Garratt, L.J. Greene, Fluorescence properties of tryptophan residues in the monomeric d-chain of *Glossoscolex paulistus* hemoglobin: an interpretation based on a comparative molecular model, Biophys. Chem. 97 (2002) 139–157.